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(54) Title: CLONING AND USES OF THE GENETIC LOCUS BCL-6			
(57) Abstract			
This invention provides methods or regulating BCL-6 levels in cells and a method of treating a subject with lymphoma.			

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CLONING AND USES OF THE GENETIC LOCUS bcl-6

5 This application claims priority of U.S. Serial No. 09/107,058, filed June 30, 1998, the content of which is hereby incorporated by reference into the subject application.

10 The invention disclosed herein was made with Government support under NIH Grant Nos. CA-44029, CA-34775, CA-08748 and CA-37295 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

15 BACKGROUND OF THE INVENTION

20 Throughout this application various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each Experimental Detail Section.

25 Non-random chromosomal abnormalities are found in up to 90% of patients with non-Hodgkin's lymphoma (NHL) and have been shown to play an important role in lymphomagenesis by activating proto-oncogenes (1). Some of these translocations, which are associated with specific histologic subsets of NHL, have been characterized at the molecular level. In the t(8;14), t(8;22), and t(2;8) translocations associated with Burkitt Lymphoma, L₃-type acute lymphoblastic leukemia and AIDS-associated non-Hodgkin lymphoma (NHL), a known proto-oncogene, c-myc, was found juxtaposed to the immunoglobulin (Ig) loci (2,3). In 30 the t(14;18) translocation, which is implicated in follicular-type NHL, molecular analysis of the sequences linked to the Ig locus led to the identification of a novel proto-oncogene, bcl-2 (4-6). The t(11;14)(q13;q32), mainly associated with "mantle zone" lymphoma, appears to involve

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the juxtaposition of the Ig heavy-chain locus with the bcl-1 locus, the site of the candidate proto-oncogene PRAD-1/cyclin D1 (7,8). These well characterized chromosome translocations are associated, however, with only a fraction of NHL cases, while a number of other recurrent translocations remain to be characterized for their genetic components.

One important example of such cytogenetic abnormalities is represented by various alterations affecting band 3q27. This region is involved in translocations with various chromosomal sites including, but not limited, to those carrying the Ig heavy-(14q32) or light-(2p12, 22q11) chain loci (9,10). Overall, 3q27 breakpoints are detectable in 7-12% of B-cell NHL cases by cytogenetic analysis, with t(3;22)(q27;q11) being the most frequent type detectable in 4-5% of NHL (9). The clinicopathologic relevance of 3q27 breakpoints is underscored by its consistent association with diffuse-type NHL, a frequent and clinical aggressive subtype for which no specific molecular lesion has yet been identified (9).

The recurrence of 3q27 breakpoints in NHL has prompted a search for the corresponding proto-oncogene. This invention discloses the cloning of clustered 3q27 breakpoints from two NHL cases carrying t(3;14)(q27;q32) translocations and the identification of genomic rearrangements within the same breakpoint region in additional NHL cases carrying translocations involving 3q27. Within the same region, a transcriptional unit has been identified, which represents the candidate proto-oncogene (bcl-6) associated with 3q27 translocations in B-NHL.

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SUMMARY OF THE INVENTION

This invention provides an isolated vertebrate nucleic acid molecule of bcl-6 locus. This invention provides an isolated vertebrate DNA molecule of bcl-6 locus. This invention provides an isolated vertebrate cDNA molecule of bcl-6. This invention provides an isolated genomic DNA molecule of bcl-6. This invention provides an isolated vertebrate RNA molecule of bcl-6. This invention provides an isolated human nucleic acid molecule of bcl-6 locus.

In addition, this invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of bcl-6.

In addition, this invention provides an isolated vertebrate DNA molecule of bcl-6 operatively linked to a promoter of RNA transcription. This invention provides a vector which comprises the isolated vertebrate DNA molecule of bcl-6.

In addition, this invention provides the above vector, wherein the isolated nucleic acid molecule is linked to a plasmid.

In addition, this invention provides a host vector system for the production of a polypeptide encoded by bcl-6 locus, which comprises the above vector in a suitable host.

In addition, this invention provides a method of producing a polypeptide encoded by bcl-6 locus, which comprises growing the above host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

In addition, this invention provides a polypeptide encoded by the isolated vertebrate nucleic acid molecule of bcl-6

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locus. Further, this invention provides an antibody capable of binding to polypeptide encoded by bcl-6 locus.

5 In addition, this invention provides an antagonist capable of blocking the expression of the polypeptide encoded by bcl-6.

10 In addition, this invention provides an antisense molecule capable of hybridizing to the nucleic acid molecule of bcl-6.

15 In addition, this invention provides an assay for non-Hodgkin's lymphoma, a method for screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma and a method for diagnosing B-cell lymphoma.

Finally, this invention provides a method of treating a subject with non-Hodgkin's lymphoma.

20 This invention further provides a method of degrading BCL-6 in cells comprising administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation.

25 This invention provides a method of treating a subject with lymphoma which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable
30 carrier, thereby treating the subject with lymphoma.

This invention provides a method of decreasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents
35 expression of BCL-6 protein so as to thereby decreasing BCL-6 levels in the cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Immunoglobulin gene rearrangement analysis of KC1445 and SM1444 DNA. DNA extracted from the cell lines U937 (monocytic leukemia) and SK-N-MC (neuroblastoma) were used as controls for non-rearranged, germ-line Ig genes. In the left panel, the arrow on the left points to the rearranged J_H fragment which does not contain C_μ sequences in KC1445 DNA, while the two arrows on the right point to the two distinct fragments containing J_H or C_μ sequences in SM1444 DNA.

Figure 2: Molecular cloning of the chromosomal breakpoints from two NHL cases with t(3;14). Illustrated are the maps of two representative phage clones spanning the breakpoint regions in case SM1444 (SM-71) and KC1445 (KC-51). Chromosome 14 portions of the phage inserts are indicated by a solid line with hatched and black boxes representing switch sequences and C_μ exons, respectively. Vertical arrows point to the junctions of chromosome 3 and 14 sequences. The probes used for Southern (Figure 4) and Northern (Figure 5) analysis are illustrated below the SM-71 map. Restriction enzyme sites are indicated as: B=*Bam*HI; H=*Hind*III; R=*Eco*RI; G=*Bbl*II; S=*sac*I.

Figure 3: Localization of phage SM-71 sequences to chromosomes 3 and 14 by fluorescence *in situ* hybridization. Consistent hybridization signals at 3q27 (arrow in panel A) and 14q32 (arrow in panel B) demonstrated that the insert is derived from the translocation junction.

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Figures 4A-4C: Southern blot hybridization analysis of bcl-6 rearrangements in NHL carrying 3q27 breakpoints. The probes used are illustrated in Figure 2. U937 and SK-N-MC DNAs are used as germ-line controls since their hybridization pattern was identical to the one observed in a panel of 19 control DNAs tested. The detected cytogenetic abnormalities affecting 3q27 in each case are: KC1445: t(3;14)(q27;q32); SM1444: t(3;14)(q27;q32); TF1403: t(3;14)(q27;q32); LD1411: t(3,14)(q27;q32); EM352: t(3;22)(q27;q11); CF755: t(3;12)(q27;q11); SO955:der(3)t(3;5)(q27;q31).

Figure 5: Identification of the bcl-6 transcriptional unit. 15 µg of total RNA isolated from the indicated human cell lines was analyzed by Northern blot hybridization using the Sac 4.0 probe (see Figure 2). CB33:EBV-immortalized human B lymphoblastoid cell line; HeLa: human cervical carcinoma cell line; Daudi: human Burkitt lymphoma cell line; Hut78: human T-cell leukemia cell line. Hybridization of the same filter to a mouse GAPDH probe is shown as control for RNA amount loaded in each lane. The faint band comigrating with 28S RNA in all the lanes may be the result of cross-hybridization with ribosomal RNA sequences.

Figure 6: Map of normal human BCL-6 locus. A recombinant genomic DNA library derived from normal placenta DNA was obtained from STRATAGENE Inc and screened by plaque hybridization using the Sac 4.0 probe. Three recombinant phages were obtained (φ 1-3 in the figure) whose inserts have been

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mapped and shown to overlap on approximately 30 kilobases of genomic DNA representing the BCL-6 locus. These sequences containing bcl-6 exons since they hybridize to the cDNA probe. The precise position of the exons has only been approximately determined and is schematically indicated in the figure. The position of the breakpoints observed in various lymphoma cases is also indicated.

Figure 7: pSac 40 plasmid construction.

Figure 8: pGB3l and pGB3s plasmid construction.

Figures 9A-9D: cDNA and Amino Acid Sequences of BCL-6 (SEQ ID NOs. 1 and 2). The Sac 4.0 probe was used to screen a recombinant phage cDNA library constructed from Bjab B cell lymphoma line RNA. A 4.0 kilobase cDNA was isolated and its nucleotide sequence was determined. It contains a long open reading frame potentially coding for 706 amino acid protein which contains five zinc-finger domains (underlined in the figure; C and H residues which identify the C2H2-type zinc-finger structure are indicated in bold).

Figures 10A-10B:

Structure of BCL-6 cDNA and sequence of its predicted protein product. Figure 10A: Schematic representation of the full-length BCL-6 cDNA clone showing the relative position of the open reading frame (box) with 5' and 3' untranslated sequences (lines flanking the box). The approximate positions of the zinc-finger motifs (Zn++) and the NH₂-terminal homology (shaded area) with other proteins are also indicated. Figure 10B: The predicted amino acid

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sequence of the BCL-6 protein. The residues corresponding to the six zinc-finger motifs (H-C links). The GenBank Accession number for BCL-6 cDNA and amino acid sequences is U00115.

Figure 11: Homology of the NH₂-terminal region of BCL-6 to other Krüppel zinc-finger proteins, viral (VA55R), or cellular non-zinc-finger (kelch) proteins. Black background indicates identical residues found four or more times at a given position; grey indicates conserved residues that appear in at least four sequences at a given position. Conserved amino acid substitutions are defined according to scheme (P, A, G, S, T), (Q, N, E, D), (H, K, R), (L, I, V, M), and (F< Y< W). Numbering is with respect to the methionine initiation codon of each gene.

Figure 12: Exon-intron organization of the BCL-6 gene and mapping of breakpoints detected in DLCL. Coding and non-coding exons are represented by filled and empty boxes, respectively. The position and size of each exon are approximate and have been determined by the pattern of hybridization of various cDNA probes as well as by the presence of shared restriction sites in the genomic and cDNA. The putative first, second and third exons have been sequenced in the portions overlapping the cloned cDNA sequences. The transcription initiation site has not been mapped (shaded box on 5' side of first exon). Patient codes (e.e. NC11, 891546 etc.) are grouped according to the rearranged patterns displayed by tumor samples. Arrows indicate the breakpoint

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position for each sample as determined by restriction enzyme/hybridization analysis. For samples KC1445 and SM1444, the breakpoints have been cloned and the precise positions are known. Restriction sites marked by asterisks have been only partially mapped within the BCL-6 locus. Restriction enzyme symbols are: S, Sac I; B, Bam HI; X, Xba I; H, Hind III; R, Eco RI; G, Bgl II; P, Pst I; Sc, Sca I; St, Stu I; Rs, Rsa I. Tumor samples were collected and analyzed for histopathology at Memorial Sloan-Kettering Cancer Center or at Columbia University.

Figures 13A-13B:

Rearrangements of the BCL-6 gene in diffuse large-cell lymphomas (DLCL). Genomic DNA extracted from tumor biopsies of DLCL cases and from normal lymphocytes (lane N) was digested with the indicated restriction enzymes and analyzed by Southern blot hybridization using the Sac 4.0 probe. Abnormal restriction fragments are indicated by the arrows.

Figures 14A-14C:

Analysis of BCL-6 rearrangements in AIDS-NHL (Figures 14A-14C). DNAs were digested with BamHI (Figure 14A) or XbaI (Figures 14B and 14C) and hybridized to probes Sac4.0 (Figures 14A and 14B) or Sac0.8 (Figure 14C). The BCL-6 germline bands detected by BamHI (11.4 Kb) and XbaI (14 Kb) are indicated. U937 was used as a BCL-6 germline control. Among the cases shown, rearrangements were detected in cases DK782, DK827, and DS16, represented by AIDS-DLCL.

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Figure 15: Restriction map of the germline *BCL-6* locus. Exon-intron organization of the *BCL-6* gene. Coding and noncoding exons are represented by filled and empty boxes, respectively. The transcription initiation site has not been mapped (shaded box on 5' side of first exon). The breakpoints detected in AIDS-NHL are indicated by arrows. Restriction enzyme symbols are: S, *SacI*; B, *BamHI*; X, *XbaI*, R, *EcoRI*. RE, restriction enzyme.

Figures 16A-16C:

Analysis of EBV infection (Figure 16A), c-MYC rearrangements (Figure 16B), and p53 mutations (Figure 16C) in AIDS-NHL. Figure 16A: Analysis of EBV termini heterogeneity in AIDS-NHL. DNAs were digested with *BamHI* and subjected to Southern hybridization using a DNA probe specific for the fused termini of the EBV genome. U937, a monocytic leukemia cell line, is used as a negative control. A lymphoblastoid cell line derived by EBV infection of normal polyclonal B cells (NC2) is used as control for polymorphic EBV termini. Representative samples of AIDS-NHL, both positive (DK3794, DK4338, DK2814, DK3973) and negative (DK3479), are shown. Figure 16B: Southern blot analysis of c-MYC rearrangements in AIDS-NHL. Genomic DNAs from the cases shown was digested with *HindIII* and probed with clone MC413RC⁴¹, representative of c-MYC exon 3. A lymphoblastoid cell line (NC2) was used as control for c-MYC germline configuration. Among the cases shown, two cases of AIDS-DLCL (DK3537 and DK1446) display a c-MYC rearrangement. Figure 16C: Analysis by PCR-SSCP of the p53 gene in

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AIDS-NHL. Representative examples are shown for p53 exon 5. Samples were scored as abnormal when differing from the normal control (N). A sample known to harbor a p53 mutation was used as positive control (POS). Among the cases shown, DK1171, a case of AIDS-SNCCL, shows a p53 mutation which was further characterized by direct sequencing of the PCR product.

Figures 17A-17B:

Southern blot analysis of the BCL-6 gene configuration in diffuse large cell lymphomas. Genomic DNA extracted from tumor biopsies was digested with the indicated restriction endonucleases and hybridized using the Sac4.0 probe (19). Rearranged fragments are indicated by the arrows. N = normal control DNA obtained from human lymphocytes.

Figures 18A-18B:

Figure 18A: Freedom from progression in BCL-6 rearranged cases (open circles, top curve) compared to BCL-6 germline cases (closed circles, bottom curve) ($P=0.007$). Figure 18B: Overall survival from time of diagnosis for BCL-6 rearranged CLLC (open circle, top curve), compared to BCL-6 germline, BCL-2 germline DLLC (dark triangles, middle curve), and BCL-2 rearranged DLLC (dark boxes, bottom curve) ($P=0.02$).

Figures 19A-19C.

Phosphorylation of BCL-6 by ERK2 *in vitro*: (Fig. 19A) Schematic representation of wild-type and mutant GST-BCL-6 fusion proteins. (*) Serines within MAPK

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phosphorylation sites (PXSP). "ZF" zinc finger domain. (Fig. 19B) ERK2 kinase assays using GST-BCL-6 wild-type and deletion mutants as substrates in the presence of [γ -³²P]-ATP. (Fig. 19C) (Top) ERK2 kinase assay for wild-type (WT) or mutant (Ala333; Ala333,343) GST-BCL6 Δ ZF proteins. (Bottom) Commassie Blue staining of the gel shown at top demonstrating comparable amounts of proteins loaded. Molecular weight markers are shown at the left.

Figures 20A-20D.

BCL-6 protein degradation induced by over-expression of MEK-2E in 293T cells. (Fig. 20A) Western blot analysis of 293T cells transfected with 5 μ g of BCL-6 (lanes 1, 3) and 5 μ g of MEK-2E (lanes 2,3) using anti-BCL-6 (N-70-6; top) or anti-ERK2 (C-14; middle) antibodies. The results of solid phase ERK2 kinase assay performed on cell extract from the same transfectants used in the top. (Fig. 20B) Western blot (top) and Northern blot (bottom) analysis of BCL-6 in 293T cells transfected with pMT2T-BCL-6 (BCL-6) (5 μ g) (lanes 1-7) and various amounts of MEK-2E-CMV (MEK-2E) (lanes 2-4) or MEK-CMV (MEK) (5, 10, or 15 μ g) (lanes 5-7) as indicated. (Fig. 20C) Western blot analysis of 293T cell extracts transfected with wild-type BCL-6 (lanes 1-3) or BCL-6_{Ala333,343} (lanes 4-6) in the absence (lanes 1, 4) or presence (2, 3, 5, 6) of cotransfected MEK-2E. (Bottom) The results of densitometric scanning of the autoradiography; analogous results were

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obtained in three independent experiments. (Fig. 20D) Analysis of BCL-6 transrepression activity in the presence of active MEK. 293T cells were transfected with 2.0 pmol of B6BS-TK-Luc, 0.04 pmol of pMT2T-BCL-6 (lanes 2-8) or pMT2T-BCL-6_{Δ333,343} (lanes 11-14) and increasing amounts (0.1, 0.2, 0.4, 0.4 pmol) of MEK-2E (lanes 3-5, 9, 12-14) or MEK (lanes 6-8, 10) as indicated. Cells were harvested 48 hrs after transfection and luciferase activities were measured by a luminometer.

Figures 21A-21B.

BCL-6 contains PEST sequences which are required for phosphorylation-induced degradation. (Fig. 21A) Schematic representation of HA-tagged BCL-6 proteins. PEST sequences were identified by the PEST-FIND program. PEST1: AA336-AA351 (KSDCQPNSPTESCSSK) (SEQ ID NO:10), score 9.4; PEST2: AA365-AA371(KSPTDPK) (SEQ ID NO:11), score 5.0; PEST3: AA406-AA430 (RAYTAPPACQPPMEPENLDLQSPK) (SEQ ID NO:12), score 2.6. (Fig. 21B) 293T cells were transfected with 5 μ g of pMT2T vectors expressing HA-BCL-6 (lanes 1-3), HA-BCL-6 Δ (300-417) (lanes 4-6), or HA-BCL-6ZF (lanes 7-9) in the absence of MEK-2E (lanes 1, 4, 7) or in the presence of increasing amount (5, 10 μ g) of MEK-2E (lanes 2, 5, 8, 3, 6, 9). Forty-eight hrs after transfection, equal amounts of cell lysates were analyzed (after normalization for transfection efficiency based on β -galactosidase activity of co-transfected

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plasmids) by 8% SDS-PAGE and Western blot using anti-HA (12CA5) antibodies.

Figures 22A-22B

5 MAPK-induced BCL-6 degradation is mediated
by the ubiquitin/proteasome pathway.
(Fig. 22A) Western blot analysis of BCL-6
10 proteins in 293T cells transfected with
BCL-6 in the absence or presence of
co-transfected MEK-2E treated with 0.2%
DMSO (lanes 1-3), 50 μ M Calpain inhibitor
II (lanes 4-6), or 50 μ M MG132 (lanes 7-9)
(added 8hrs after transfection). (Fig.
22B) 293T cells were transfected with
15 BCL-6, His₆-Ub, and MEK-2E in the absence
(lanes 1-4) or presence of MG132 (lanes
5-8). Cell lysates were immunoprecipitated
with anti-BCL-6 antibodies (N-70-6) and
the immunoprecipitants were analyzed by 6%
20 SDS-PAGE followed by Western blot analysis
using anti-ubiquitin antibodies.

Figures 23A-23E.

25 BCL-6 is phosphorylated and degraded by
antigen receptor signaling in B cells.
Ramos cells (1×10^6 /ml) were treated with
anti-IgM (10 g/ml) and harvested at
different time points after treatment as
indicated. (Fig. 23A) (Top three panels)
30 Equal amounts of cell extracts were used
for Western blot analysis using
anti-BCL-6 (top), or anti-ERK2 (middle)
antibodies, and for solid phase ERK2
kinase assays (MBP, bottom); Equal
35 amounts of RNAs (10 μ g) were used for
Northern blot analysis with BCL-6 or GAPDH
probes (bottom). (Fig. 23B)
Hyperphosphorylated BCL-6 proteins are

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more unstable. Ramos cells were pulse labeled for 1 hour with [³⁵S]methionine and [³⁵S]cysteine, and then treated with anti-IgM (10 μ g/ml) for 30 min and subsequently incubated in the presence of an excess of nonradioactive methionine and cysteine for the indicated times (chase). Cell extracts were immunoprecipitated with anti-BCL-6 antibodies and analyzed by SDS-PAGE followed by autoradiography. (Fig. 23C) Anti-IgM induced BCL-6 phosphorylation and degradation is prevented by a specific MEK inhibitor. Western blot analysis of BCL-6 in Ramos cells treated with anti-IgM in the presence of 0.2% DMSO or 50 μ M PD098059 (added 30 min before anti-IgM treatment). (Fig. 23D) Anti-IgM induced BCL-6 degradation is prevented by a specific proteasome inhibitor. Western blot analysis of BCL-6 in Ramos cells treated with anti-IgM in the presence of 0.2% DMSO (lanes 2-4), 50 μ M Calpain inhibitor II (lanes 5-7), and 50 μ M MG132 (lanes 8-10) (added 1 hr before the treatment). (Fig. 23E) Mutant BCL-6 proteins are resistant to anti-IgM-induced degradation. Ramos cells stably transfected with pHeBo-MT-HA-BCL6, pHeBo-MT-HA-BCL-6_{Ala333,343} and pHeBo-MT-HA-BCL6ZF were treated with 1 μ M CdCl₂ for 6 hrs to induce exogenous BCL-6 expression. Cells were then treated with anti-IgM (10 μ g/ml) and harvested at different time points as indicated. Equal amounts of cell extracts were loaded on 7% (HA-BCL-6 or HA-BCL-6_{Ala333,343}) or 10% SDS-PAGE (HA-BCL-6ZF) and the amount of exogenous BCL-6 proteins were analyzed by

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Western blot using anti-HA antibodies
(12CA5).

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DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

5 C=cytosine A=adenosine
 T=thymidine G=guanosine

10 This invention provides an isolated vertebrate nucleic acid molecule of the bcl-6 locus. As used herein, bcl-6 locus means the breakpoint cluster region in B-cell lymphomas. The bcl-6 locus is of 30 kilobase in length containing at least a bcl-6 gene which codes for a protein. Therefore, the bcl-6 locus contains both the 5' and 3' flanking region of the coding sequences of the bcl-6 gene.

15 In an embodiment, the isolated, vertebrate nucleic acid molecule of bcl-6 locus is DNA. In another embodiment, the isolated, vertebrate nucleic acid of the bcl-6 locus is cDNA. In a further embodiment, the isolated, vertebrate
20 nucleic acid is genomic DNA. In a still further embodiment, the isolated, vertebrate nucleic acid molecule is RNA.

25 This invention provides an isolated, human nucleic acid molecule comprising the bcl-6 locus.

30 The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host
35 cells capable of expression of the polypeptide and related products.

Moreover, the isolated vertebrate nucleic acid molecules

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are useful for the development of probes to study B cell lymphomas.

5 This invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the bcl-6 locus. In an embodiment, this molecule is DNA. In another embodiment, the molecule is RNA.

10 As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between
15 complementary base pairs.

The above nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of bcl-6 locus may be used as a probe for bcl-6 sequences.
20 Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe
25 molecules may be produced by insertion of a DNA molecule having the full-length or a fragment of the bcl-6 locus into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells
30 and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

35 RNA probes may be generated by inserting the full length or a fragment of the bcl-6 locus downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with a linearized bcl-6 or its fragment where it contains an

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upstream promoter in the presence of the appropriate RNA polymerase.

5 This invention provides an cDNA molecule of bcl-6 locus operatively linked to a promoter of RNA transcription.

10 This invention provides a vector which comprises the nucleic acid molecule of bcl-6 locus. This invention provides the above vector, wherein the isolated nucleic acid molecule is linked to a plasmid.

15 This invention further provides isolated cDNA molecule of the bcl-6 locus operatively linked to a promoter of RNA transcription. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners.

20 As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which
25 cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

30 In an embodiment, a partial cDNA molecule of the bcl-6 locus is linked to pGEM-7zf(-) and the resulting plasmid is designated as pGB31 (Figure 8). Plasmid, pGB31 was deposited on June 3, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of
35 Microorganism for the Purposes of Patent Procedure. Plasmid, pGB31 was accorded with ATCC Accession Number 75476.

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In an another embodiment, a partial cDNA molecule of the bcl-6 locus is linked to pGEM-7zf(-) and the resulting plasmid is designated as pGB3s (Figure 8). Plasmid, pGB3s was deposited on June 3, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, pGB3s was accorded with ATCC Accession Number 75477.

This invention provides a host vector system for the production of a polypeptide encoded by bcl-6 locus, which comprises the above vector in a suitable host.

This invention provides the above host vector system, wherein the suitable host is a bacterial cell, insect cell, or animal cell.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal; the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the polypeptide encoded by the bcl-6 locus.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is

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selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention provides a method of producing a polypeptide encoded by bcl-6 locus, which comprises growing the above host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a polypeptide encoded by the isolated vertebrate nucleic acid molecule of bcl-6 locus.

This invention provides an antibody capable of binding to polypeptide encoded by bcl-6 locus. In an embodiment, the antibody is monoclonal.

This invention provides a method to select specific regions on the polypeptide encoded by the bcl-6 locus to generate antibodies. The protein sequence may be determined from the cDNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to polypeptide encoded by the bcl-6 locus. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and

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used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides.

5 Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be
10 produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of polypeptide encoded by the bcl-6 locus in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

15 The antibody may be labelled with a detectable marker, including but not limited to: a radioactive label, or a calorimetric, luminescent, or fluorescent marker, or gold. Radioactive labels include but are not limited to: ^3H , ^{14}C ,
20 ^{32}P , ^{33}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{59}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re . Fluorescent markers include but are not limited to: fluorescein, rhodamine and auramine. Methods of producing the polyclonal or monoclonal antibody are known to one of ordinary skill in the art.

25 Further, the antibody complex may be detected by a second antibody which may be linked to an enzyme, such as alkaline phosphatase or horseradish peroxidase. Other enzymes which may be employed are well known to one of ordinary skill in the art.

30 This invention provides for the isolated nucleic acid molecule of bcl-6 that is labelled with a detectable marker. The detectable marker may be a radioactive label, a calorimetric, luminescent, or a fluorescent marker.
35 Other detectable markers are known to those skilled in the art as hereinabove described.

This invention provides an antagonist capable of blocking

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the expression of the polypeptide encoded by the isolated nucleic acid molecule of bcl-6. The antagonist may be a triplex oligonucleotide capable of hybridizing to nucleic acid molecule bcl-6.

5

This invention provides an antisense molecule capable of hybridizing to the nucleic acid molecule bcl-6. The antisense molecule may be DNA or RNA.

10

This invention provides a triplex oligonucleotide capable of hybridizing with a double stranded DNA molecule bcl-6.

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The antisense molecule may be DNA or RNA or variants thereof (i.e. DNA with a protein backbone). The present invention extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of the receptor recognition proteins at the translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

20

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Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules upon introduction to cells.

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This invention provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule bcl-6 introduced into the mammal at an embryonic stage.

This invention provides an assay for non-Hodgkin's

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lymphoma, comprising (a) incubating a sample of suitable body fluid for a subject with a monoclonal antibody reactive with non-Hodgkin's lymphoma cells to a solid support, (b) removing unbound body fluid from the support, and (c) determining the level of antigen activity exhibited by the bound body fluid to the support.

The suitable bodily fluid sample is any bodily fluid sample which would contain non-hodgkin lymphoma cells or fragments thereof. A suitable bodily fluid includes, but is not limited to, serum, plasma, cerebrospinal fluid, and urine. In the preferred embodiment, the suitable bodily fluid sample is serum or plasma. In addition, the body fluid sample may cells from bone marrow, or a supernate from a cell culture. Methods of obtaining a suitable bodily fluid sample from a subject are known to those skilled in the art.

This invention provides a method for screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma, which comprises determining in a first sample from a subject with non-Hodgkin's lymphoma the presence of the isolated nucleic acid molecule bcl-6, administering to the subject a therapeutic amount of the agent such that the agent is contacted with the cell associated with the condition, determining after a suitable period the amount of the isolated nucleic acid molecule in a sample from the treated subject, and comparing the amount of isolated nucleic acid molecule determined in the first sample with the amount determined in the sample from the treated subject, a difference indicating the effectiveness of the agent, thereby screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma.

Further, this invention provides an assay system that is employed to identify drugs or other molecules capable of binding to the nucleic acid molecule bcl-6 or proteins, either in the cytoplasm or in the nucleus, thereby

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inhibiting or potentiating transcriptional activity. Such assay would be useful in the development of drugs that would be specific against particular cellular activity, or that would potentiate such activity, in time or in level of activity.

The above described probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues.

The in-situ hybridization technique using the labelled nucleic acid molecule bcl-6 is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker. Further, immunohistochemical protocols may be employed which are known to those skilled in the art.

This invention provides a method of diagnosing diffuse-type B-cell lymphoma in a subject which comprises detecting in a sample from the subject nucleic acid molecule of bcl-6 locus.

This invention provides a method for diagnosing B-cell lymphoma in a subject comprising: (a) obtaining DNA sample from the subject; (b) cleave the DNA sample into fragments; (c) separating the DNA fragments by size fractionation; (d) hybridizing the DNA fragments with a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of the bcl-6 locus to detect the DNA fragment containing the bcl-6 sequence; and (e) comparing the

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detected DNA fragment from step (d) with the DNA fragment from a known normal subject, the difference in size of the fragments indicating the occurrence of B-cell lymphoma in the subject. In a preferred embodiment, the above
5 diagnostic method is for diffuse-type B-cell lymphomas.

A person of ordinary skill in the art will be able to obtain appropriate DNA sample for diagnosing B-cell lymphoma in a subject. The DNA sample obtained by the
10 above described method may be cleaved by restriction enzyme. The uses of restriction enzymes to cleave DNA and the conditions to perform such cleavage are well-known in the art.

15 In an embodiment, the size fractionation in step (c) of the above-described method is effected by a polyacrylamide gel. In another embodiment, the size fractionation is effected by an agarose gel.

20 This invention also provides the above-described diagnosis method wherein step the nucleic acid molecule in step (d) is labeled with a detectable marker. The detectable marker includes but is not limited to a radiolabelled molecule, a fluorescent molecule, an enzyme, or a ligand.

25 In a preferred embodiment, the above-described diagnosis method further comprises transferring the DNA fragments into a solid matrix before the hybridization step (d). One example of such solid matrix is nitrocellulose paper.

30 As an example for the above-described diagnosis method is shown in Figures 4A-4C where different NHL sample are analyzed. More lymphoma cases and their breakpoints are shown in Figure 6.

35 This invention also provides a method for diagnosing B-cell lymphoma in a subject comprising: (a) obtaining RNA sample from the subject; (b) separating the RNA sample into

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different species by size fractionation; (c) hybridizing the RNA species with a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of the bcl-6 locus to detect the RNA species containing the bcl-6 sequence; and (d) comparing the RNA species obtained from (c) with the RNA species from a known normal subject, the difference in size of the species indicating the occurrence of B-cell lymphoma in the subject.

In an embodiment, the size fractionation in step (b) is effected by a polyacrylamide or agarose gel.

This invention also provides the above-described method where in step (c), the nucleic acid molecule is labeled with a detectable marker. The detectable marker includes but is not limited to a radiolabelled molecule, a fluorescent molecule, an enzyme, or a ligand.

This invention also provides the above-method further comprises transferring the RNA species into a solid matrix before step (c).

This invention also provides various uses of bcl-6 locus/gene and its derivatives. This invention further provides a method for diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using bcl-6 DNA probes or synthetic oligonucleotide primers derived from bcl-6 sequences to detect bcl-6 rearrangements/mutations by Southern blotting PCR or other DNA based techniques.

This invention also provides a method of diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using bcl-6 DNA probes or synthetic oligonucleotide primers derived from bcl-6 sequences to detect abnormal bcl-6 RNA species by Northern blotting, PCR or other RNA-based techniques.

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This invention further provides a method of diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using antiserum or monoclonal antibodies directed against the bcl-6 protein product(s).

5

This invention provides a method of treating a subject with non-Hodgkin's lymphoma comprising administering an effective amount of the antisense molecule of the nucleic acid molecule bcl-6 operatively linked to a suitable regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby treating the subject with non-Hodgkin's lymphoma.

10

This invention provides a method of treating a subject with non-Hodgkin's lymphoma, comprising administering an effective amount of the antagonist capable of blocking the expression of the polypeptide encoded by the isolated nucleic acid molecule of bcl-6, and a suitable acceptable carrier, thereby treating the subject with non-Hodgkin's lymphoma.

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Further, as is known to those of ordinary skill in the art effective amounts vary with the type of therapeutic agent. It is known to those of ordinary skill in the art how to determine an effective amount of a suitable therapeutic agent.

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The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose,

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glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The subjects contained herein may be a mammal, or more specifically a human, horse, pig, rabbit, dog, monkey, or rodent. In the preferred embodiment the subject is a human.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration.

As used herein administration means a method of

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administering to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intramuscularly, subcutaneously or by aerosol. Administration of the agent may be effected continuously or intermittently such that the therapeutic agent in the patient is effective to treat a subject with non-hodgkin's lymphoma.

Finally, this invention provides a therapy of B cell lymphoma and/or diffuse-type B cell lymphoma using anti bcl-6 reagents including specific antisense sequences and compounds interfering with bcl-6 functions.

This invention further provides a method of degrading BCL-6 in cells comprising administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation. In an embodiment of the above-described method of the molecule which induces phosphorylation of the BCL-6 is a mitogen-activated protein kinase (MAPK). In another embodiment of the method of the molecule which induces phosphorylation of the BCL-6 is a functionally active mutant of a mitogen-activated protein kinase (MAPK). In an embodiment of the method the MAPK includes but is not limited to ERK-1 or ERK-2. In an embodiment the BCL-6 is phosphorylated either at one phosphorylation site or at multiple sites. In a preferred embodiment of the method, the molecule which induces phosphorylation of the BCL-6 is a molecule which activates an antigen receptor on B cell surfaces. In an embodiment the molecule which activates an antigen receptor on B cell surfaces is an antibody. In an embodiment the antibody includes but is not limited to an anti-IgM antibody. The antibody may also be an anti-idiotypic antibody which activates an antigen receptor on B cell surfaces. In another embodiment of the above-described method the molecule which activates an antigen receptor on B cell surfaces is a molecule which activates MAPK in B cells. As used herein "activation of an antigen

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receptor on B cell surfaces" is defined as induction of signal transduction including MAPK activation. In a preferred embodiment of the method the molecule which activates MAPK in B cells is a cytokine. In a further
5 preferred embodiment of the method the cytokine used includes but is not limited to TNF, IL-6, or IL-2. In an embodiment of the method the molecule is cross-linked to a B cell antigen receptor to activate the receptor. In an
10 embodiment cross-linking the molecule to the B cell antigen receptor activates the MAPK.

This invention provides a method of treating a subject with lymphoma which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which
15 induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable carrier, thereby treating the subject with lymphoma. In an embodiment of the above-described method the lymphoma expresses BCL-6. In a preferred embodiment of the method
20 the pharmaceutical composition comprises a MAPK activator. In a further preferred embodiment the MAPK activator is an antibody. In another embodiment of the method the antibody used includes but is not limited to an anti-IgM antibody. The antibody may also be an anti-idiotypic antibody which
25 activates an antigen receptor on B cell surfaces. In another embodiment the MAPK activator is a cytokine. The cytokine used may be selected from but is not limited to an cytokines such as TNF, IL-6, or IL-2. In a preferred embodiment the lymphoma is a B-cell lymphoma. In another
30 preferred embodiment the B-cell lymphoma is derived from germinal center B cells. In an embodiment of the above-described method the administration of the pharmaceutical composition may be selected from but is not limited to intravenous or intratumor administration.

35 This invention provides a method of decreasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents

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expression of BCL-6 protein so as to thereby decreasing BCL-6 levels in the cells. In an embodiment of the above-described method the compound which interferes with transcription of bcl-6 prevents binding of a transcription factor and histone acetylase/deacetylase complexes.

In another embodiment the compound is N,N'-hexamethylene bisacetamide (HMBA) or trichostatin. Applicants incorporate by reference U.S. Patent 5,608,108 issued March 4, 1997 (Marks et al.), U.S. Patent 5,175,191 issued December 29, 1992 (Marks et al.), and U.S. Patent 5,055,608 issued October 8, 1991 (Marks et al.) for compounds disclosed therein which interfere with transcription. In an embodiment the method of treating lymphoma comprising decreasing BCL-6 levels in cells comprises the above-described method of decreasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents expression of BCL-6 protein so as to thereby decreasing BCL-6 levels in the cells.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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EXPERIMENTAL DETAIL SECTION I:**Materials and Methods**

5 **DNA Extraction and Southern Blot Analysis.** Total genomic
DNA was purified from frozen tumor biopsies by cell lysis,
proteinase K digestion, "salting-out" purification and
ethanol precipitation as previously described (11).
10 Southern blot hybridization analysis was performed in 50%
formamide, 3X SSC, 10X dextran sulphate, 5X Denhardt's
solution, 0.5% SDS at 37°C for 16 hrs. Filters were washed
in 0.2X SSC, 0.5% SDS at 60°C for 2 hrs. DNA probes were
32P-labelled by the random priming method (12).

15 **DNA Probes.** The following probes were used for Southern
blot analysis of Ig gene rearrangements: i) (J_H) probe:
6.6 kb *Bam*HI/*Hind*III fragment from the human Ig heavy-chain
(Ig_H) locus (13); ii) (C_μ) probe: 1.3 kb *Eco*RI fragment
containing the first two exons of human C_μ (13).

20 **Genomic Cloning.** Genomic libraries from NHL cases SM1444
and KC1445 were constructed by partial *Sau* 3A restriction
digestion of genomic DNA and ligation of gel-purified 15-20
kb fractions into LambdaGem-11 phage vector (Promega).
25 Library screening was performed by plaque-hybridization
using the C_μ probe.

30 **Fluorescence in situ Hybridization Analysis (FISH).** Phage
DNA was labelled with biotin-14-dATP by nick translation
and hybridized to metaphase spreads from normal human
lymphocytes as described (14). To visualize the
hybridization signal and the corresponding bands
sequentially under the microscope, the slides were stained
and counterstained with propidium iodide and 4'6'-
35 diamideno-2-phenylindole (DAPI), respectively.

Northern Blot Hybridization Analysis. RNAs from several
human cell lines were extracted by the guanidine-

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isothiocyanate method (15). For Northern blot analysis, RNA samples were electrophoresed through 0.9% agarose-2.2M formaldehyde gels and then transferred to nitrocellulose filters. Hybridization and washing were performed as described for Southern blot analysis.

Experimental Results:

DNA was extracted from tumor tissue of two cases (SM1444 and KC1445) of IgM-producing, diffuse-type B-cell NHL carrying the t(3;14)(q27;q32) translocation. Since the involvement of the Ig_H locus was suspected based on the 14q32 breakpoint, SM1444 and KC1445 DNAs were first analyzed by Southern blot hybridization using combinations of enzymes and probes specific for the J_H and C_μ regions of the Ig_H locus (13). In both cases, digestion by BamHI showed rearranged fragments containing J_H sequences (Figure 1). Subsequent hybridizations to the C_μ probe showed, in each case, that one rearranged fragment containing J_H sequences was not linked to C_μ sequences (see failure of the C_μ probe to hybridize to the same rearranged BamHI fragment detected by J_H (Figure 1) as would be expected for the physiologically rearranged Ig_H allele in IgM producing cells. In addition, in both cases, digestion with HindIII and hybridization with C_μ detected a rearranged fragment, a finding inconsistent with either germ-line or physiologically rearranged Ig_H genes, since both HindIII sites flanking C_μ sequences are not involved in V-D-J arrangements (13). The observed pattern is, however, consistent with chromosomal breakpoints located within C_μ switch sequences, as previously observed in several cases of chromosomal translocations involving the Ig_H locus (2,16-18).

Based on this analysis, the C_μ containing fragments from each case were cloned by screening genomic libraries constructed from SM1444 and KC1445 DNAs using the C_μ probe. Restriction mapping and hybridization analysis of several

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phage clones led to the identification of recombinant phages from each library which contained C_{μ} sequences linked to sequences unrelated to the Ig_H locus (see Figure 2 for maps of representative phage clones). The Ig portions of the phage inserts overlapped along the C_{μ} region extending 5' into the switch region where alignment with the restriction map of the normal Ig heavy-chain locus was lost. The location of the breakpoint within C_{μ} switch sequences was confirmed for case SM1444 by DNA sequence analysis of the breakpoint junction of phage SM-71, which revealed the presence of the repeated motifs typical of the Ig_H switch regions on the chromosome 14 side (19). The Ig -unrelated portions of phage SM-71 and KC-51 also overlapped with each other in their restriction maps, suggesting that they were derived from the same genomic region. This notion is further supported by the fact that probe Sac 4.0 derived from SM-71 was able to hybridize to the corresponding region of KC-51 in Southern blot analysis.

To determine the chromosomal origin of the Ig -unrelated sequences, a recombinant phage (SM-71) derived from case SM1444, was used as a probe in FISH analysis on metaphase chromosome spreads from mitogen-stimulated normal blood lymphocytes. The phage probe hybridized specifically to chromosome 14q32 as well as to chromosome 3q27 (Figure 3), indicating that the recombinant phage insert contained one of the two chromosomal junctions of the reciprocal $t(3;14)$ translocation. Thus, taken together, the results of cloning and FISH analysis established that, in both NHL cases studied, the chromosomal translocation has linked sequences within the switch region of the C_{μ} locus to sequences from band 3q27, consistent with the cytogenetic description of the $t(3;14)(q27;q32)$ translocation. In the two NHL cases studied, the breakpoints on 3q27 were located within 3 kb of the same genomic locus, which was termed bcl-6.

In order to determine whether 3q27 breakpoints in

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additional NHL cases were also located within the cloned portion of the bcl-6 locus, bcl-6 rearrangements were examined in a total of 19 NHL cases carrying 3q27 breakpoints, including 4 (two cloned cases and two additional ones) carrying t(3;14)(q27;q32) as well as 15 cases carrying 3q27 translocations involving regions other than 14q32. Southern blot hybridization using probes derived from phage SM-71 (see Figure 2) detected rearranged fragments in *EcoRI*-and/or *BglII*-digested DNA in 7 of 19 cases studied, including all 4 t(3;14) cases as well as 3 cases with other types of translocations (see Figures 4A-4C for cytogenetic description of the cases and representative results). These results indicate that heterogeneous 3q27 breakpoints cluster in a fairly restricted region within bcl-6 independently of the partner chromosome involved in the translocation.

Whether the bcl-6 locus adjacent to the chromosomal breakpoints contained a transcriptional unit was investigated. Probe Sac 4.0 (see Figure 2) was used to detect RNA expression in several human cell lines by Northern blot analysis. A major 2.4 kb RNA species was readily detectable in two B-cell derived cell lines tested, while a relatively less abundant 4.4 kb species is present in CB33 only. No hybridization was detected in a T-cell derived cell line (HUT 78) nor in HeLa cells (Figure 5). This result indicates that 3q27 sequences immediately adjacent to the chromosomal breakpoint cluster are part of a gene (bcl-6) which is expressed in cells of the B lineage.

Experimental Discussion:

This study reports the identification and cloning of a genomic region, bcl-6, involved in recurrent chromosomal translocations affecting band 3q27 in NHL. The region is defined by the clustered position of breakpoints in seven NHL cases carrying 3q27 translocations involving either IgH

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or several other loci. A more precise definition of the bcl-6 locus and of the frequency of its involvement in NHL requires cloning and characterization of additional bcl-6 sequences and studying additional tumor cases. Nevertheless, the finding that various translocation partner chromosomes have been joined to the same region on chromosome 3 in cytogenetically heterogenous NHL cases supports the notion that rearrangement of the bcl-6 locus may represent the critical common denominator of translocations involving 3q27.

The second finding of this study is that the bcl-6 locus contains a gene which is expressed in B-cells. It is not clear at this stage whether the chromosomal breakpoints directly truncate coding or regulatory sequences of bcl-6, or, whether the gene remains intact with its regulation overridden by transcriptional control motifs juxtaposed by the translocation. The clustering of breakpoints in the seven studied NHL cases suggests, however, that bcl-6 may be a proto-oncogene which can contribute to NHL pathogenesis upon activation by chromosomal translocation. Results of this study will allow elucidation of the normal structure and function of the bcl-6 gene in order to understand the pathogen consequences of chromosomal translocation of bcl-6 and its role in lymphomagenesis.

REFERENCES FOR SECTION I:

1. Gaidano, G., and Dalla-Favera, R., (1992) Oncogenes and tumor suppressor genes. In: Neoplastic Hematopathology, D.M. Knowles (ed.), Wilkins & Wilkins, pp 245-261.
2. Dalla-Favera, R., et al. (1982) Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells, Proc. Natl. Acad. Sci. USA 79:7824-7827.

-38-

3. Taub, R., et al. (1982) Translocation of c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells, Proc. Natl. Acad. Sci. USA 79:7837-7841.
- 5 4. Bakhshi, A., et al. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around J_H on chromosome 14 and near a transcriptional unit on 18, Cell 41:889-906.
- 10 5. Tsujimoto, U., et al. (1985) Involvement of the Bcl-2 gene in human follicular lymphoma, Science 228:1440-1443.
- 15 6. Cleary, M.L., and Sklar, J., (1985) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18, Proc. Natl. Acad. Sci. USA 82:7439-20 7444.
7. Motokura, T., et al. (1991) A novel cyclin encoded by a bcl-1 linked candidate oncogene, Nature 350:512-514.
- 25 8. Raffeld, M., and Jaffe, E.S., (1991) Bcl-1, t(11;14), and mantle zone lymphomas, Blood 78:259-261.
9. Offit, K., et al. (1989) t(3;22)(q27;q11): A novel translocation associated with diffuse non-Hodgkin's lymphoma, Blood 74:1876-1879.
- 30 10. Bastard, C., et al. (1992) Translocations involving band 3q37 and Ig gene regions in non-Hodgkin's lymphoma, Blood 79:2527-2531.
- 35 11. Miller, S.A., et al. (1988) A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res, 16:1215-1218.

-39-

12. Feinberg, A.P., and Vogelstein, B., (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity, Anal. Biochem., 132:6-13.
- 5
13. Ravetch, J.V., et al. (1981) Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D regions, Cell, 27:583-591.
- 10
14. Rao, P.H., et al. (1994) Subregional localization of 20 single-copy loci to chromosome 6 by fluorescence in situ hybridization, Cyto. and Cell Genetics 66:272-273.
- 15
15. Chirgwin, J.M., et al. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, Biochemistry, 18:5294-5299.
- 20
16. Peschle, C., et al. (1984) Translocation and rearrangement of c-myc into immunoglobulin alpha heavy chain locus in primary cells from acute lymphocytic leukemia, Proc. Natl. Acad. Sci. U.S.A., 81:5514-5518.
- 25
17. Showe, L.C., et al. (1985) Cloning and sequencing of a c-myc oncogene in a Burkitt's lymphoma cell line that is translocated to a germ line alpha switch region, Mol. Cell. Biol., 5:501-509.
- 30
18. Neri, A., Barriga, et al. (1988) Different regions of the immunoglobulin heavy chain locus are involved in chromosomal translocations in distinct pathogenic forms of Burkitt lymphoma, Proc. Natl. Acad. Sci. USA, 85:2748-2752.
- 35
19. Rabbits, T.H., et al. (1991) Human immunoglobulin heavy chain genes: evolutionary comparisons of C μ , C δ and C γ genes and associated switch sequences, Nucleic Acids Res., 19:4509-4524.

-40-

20. Schmid, et al. (1991) Nature, 332:733.

-41-

EXPERIMENTAL DETAIL SECTION IIIntroduction:

5 The molecular analysis of specific chromosomal
translocations has improved the understanding of the
pathogenesis of non-Hodgkin lymphoma (NHL), a heterogeneous
group of B-cell or, less frequently, T-cell malignancies
(1,2). The (14;18) chromosomal translocation, which causes
10 the deregulated expression of the anti-apoptosis gene BCL-
2, plays a critical role in the development of follicular
lymphoma (FL) (3-6), which accounts for 20 to 30% of all
NHL diagnoses (7). Burkitt's lymphoma (BL) and mantle-cell
lymphoma, two relatively rare NHL types, are characterized
15 by chromosomal translocations causing the deregulated
expression of the cell-cycle progression genes C-MYC and
the BCL-1/cyclin D1, respectively (8-15).

20 Relatively little is known about the molecular pathogenesis
of diffuse large cell lymphoma (DLCL), the most frequent
and most lethal human lymphoma (7). DLCL accounts for ~40%
of initial NHL diagnoses and is often the final stage of
progression of FL(7). A small percentage of DLCL display
C-MYC rearrangements (16) and 20 to 30% display alterations
25 of BCL-2 reflecting the tumor's derivation from FL (17).
However, no consistent molecular alteration has been
identified that is specific for DLCL.

30 Chromosomal translocations involving reciprocal
recombinations between band 3q27 and several other
chromosomal sites are found in 8 to 12% of NHL cases,
particularly in DLCL (18-19). From NHL samples displaying
recombinations between 3q27 and the immunoglobulin (Ig)
heavy chain locus on 14q32, the chromosomal junctions of
35 several (3;14)(q27;q32) translocations were cloned and
identified a cluster of breakpoints at a 3q27 locus named
BCL-6.

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Experimental Results:

To isolate normal BCL-6 cDNA, a cDNA library constructed from the NHL cell line Bjab (22) was screened with a probe (20-21) derived from the chromosomal region flanking the breakpoints of two t(3;14)(q27;32) cases. A phage cDNA library constructed from RNA of the Bjab lymphoma cell line was screened (1×10^6 plaques) by plaque hybridization with the Sac 4.0 probe that had been ^{32}P -labelled by random priming (22). Sequence analysis (Figures 10A-10B) revealed that the longest clone (3549 bp), approximately the same size as BCL-6 RNA, codes for a protein of 706 amino acids with a predicted molecular mass of 79kD. The putative ATG initiation codon at position 328 is surrounded by a Kozak consensus sequence (23) and is preceded by three upstream in-frame stop codons. The 1101-bp 3'-untranslated region contains a polyadenylation signal followed by a track of poly(A). These features are consistent with BCL-6 being a functional gene.

The NH_2 - and COOH - termini of the BCL-6 protein (Figures 10A-10B) have homologies with "zinc-finger" transcription factors (24). BCL-6 contains six C_2H_2 zinc-finger motifs (Figure 10A) and a conserved stretch of six amino acids (the H/C link) connecting the successive zinc-finger repeats (25). BCL-6 can be assigned to the Krüppel-like subfamily of zinc-finger proteins. The NH_2 - terminal region of BCL-6 is devoid of the FAX (27) and KRAB (28) domains sometimes seen in Krüppel-related zinc-finger proteins, but it does have homologies (Figure 11) with other zinc-finger transcription factors including the human ZFPJS protein, a putative human transcription factor that regulates the major histocompatibility complex II promoter, the Tramtrack (ttk) and Broad-complex (Br-c) proteins in Drosophila that regulate developmental transcription (29), the human KUP protein (31), and the human PLZF protein, which is occasionally involved in chromosomal translocations in human promyelocytic leukemia (32). The regions of NH_2 -

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terminal homology among ZFPJS, ttk, Br-c, PLZF and BCL-6 also share some degree of homology with viral proteins (e.g. VA55R) of the poxvirus family (33) as well as with the Drosophila kelch protein involved in nurse cell-oocyte interaction (34). These structural homologies suggest that BCL-6 may function as a DNA-binding transcription factor that regulates organ development and tissue differentiation.

The cDNA clone was used as a probe to investigate BCL-6 RNA expression in a variety of human cell lines by Northern blot analysis. A single 3.8 kb RNA species was readily detected (Figure 11) in cell lines derived from mature B-cells, but not from pro-B-cells or plasma cells, T cells or other hematopoietic cell lineages. The BCL-6 RNA was not detectable in other normal other tissues, except for skeletal muscle in which low level expression was seen. Thus, the expression of BCL-6 was detected in B-cells at a differentiation stage corresponding to that of DLCL cells. This selective expression in a "window" of B-cell differentiation suggests that BCL-6 plays a role in the control of normal B-cell differentiation and lymphoid organ development.

To characterize the BCL-6 genomic locus, the same cDNA probe to screen a genomic library from human placenta was used. A phage genomic library constructed from normal human placenta DNA (Stratagene) was screened (8×10^5 plaques) with the BCL-6 cDNA. Twelve overlapping clones spanning ~50kb of genomic DNA were isolated. After restriction mapping, the position of various BCL-6 exons was determined by Southern hybridization using various cDNA probes. By restriction mapping, hybridization with various cDNA probes, and limited nucleotide sequencing, the BCL-6 gene was found to contain at least ten exons spanning ~26 kb of DNA (Figure 12). Sequence analysis of the first and second exons indicated that they are noncoding and that the translation initiation codon is within the third exon.

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Various cDNA and genomic probes were used in Southern (DNA) blot hybridizations to determine the relationship between 3q27 (Table 1). Monoallelic rearrangements of BCL-6 were detected in 12 of 17 tumors by using combinations of restriction enzymes (Bam HI and Xba I) and probe which explore ~16 kb within the BCL-6 locus. These 12 positive cases carry recombinations between 3q27 and several different chromosomes (Table 1), indicating that heterogeneous 3q27 breakpoints cluster in a restricted genomic locus irrespective of the partner chromosome involved in the translocation. Some DLCL samples (5 of 17) do not display BCL-6 rearrangements despite cytogenetic alterations in band 3q27, suggesting that another gene is involved or, more likely, that there are other breakpoint clusters 5' or 3' to BCL-6. If the latter is true, the observed frequency of BCL-6 involvement in DLCL (33%, see below) may be an underestimate.

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Table 1.

Frequency of BCL-6 rearrangements in DLCL carrying chromosomal translocations affecting band 3q27

5	Translocation	Fraction of tumors with BCL-6 rearrangements
10	t(3;14)(q27;q32)	4/4
	t(3;22)(q27;q11)	2/3
	t(3;12)(q27;q11)	1/1
	t(3;11)(q27;q13)	1/1
	t(3;9)(q27;p13)	0/1
	t(3;12)(q27;q24)	0/1
15	der(3)t(3;5)(q27;q31)	1/1
	t(1;3)(q21;q27)	1/1
	t(2;3)(q23;q27)	1/1
	der(3)t(3;?)(q27;?)	1/3
20	Tumor samples listed in the Table were collected and analyzed for histopathology and cytogenetics at Memorial Sloan-Kettering Cancer Center.	
25	A panel of tumors not previously selected on the basis of 3q27 breakpoints but representative of the major subtypes of NHL as well as of other lymphoproliferative diseases was analyzed. Similar rearrangements were detected in 13 of 39 DLCL, but not in other cases including other NHL subtypes (28 FL, 20 BL, and 8 small lymphocytic NHL), acute lymphoblastic leukemia (ALL; 21 cases), and chronic lymphocytic leukemia (CLL; 31). These findings indicate that BCL-6 rearrangements are specific for and frequent in DLCL. In addition, the frequency of rearrangements in DLCL (33%) significantly exceeds that (8 to 12%) reported at the cytogenetic level, suggesting that some of the observed rearrangements may involve submicroscopic chromosomal alterations undetectable at the cytogenetic level.	
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All the breakpoints in BCL-6 mapped to the putative 5' flanking region, the first exon or the first intron (Figure 12). For two patients that carry (3;12)(q27;q32) translocations, the chromosomal breakpoints have been cloned and precisely mapped to the first intron (SM1444) or to 5' flanking sequences (KC1445) of BCL-6 on 3q27, and to the switch region of IgH on 14q32 (20-21). In all rearrangements, the coding region of BCL-6 was left intact whereas the 5' regulatory region, presumably containing the promoter sequences, was either completely removed or truncated. The resultant fusion of BCL-6 coding sequences to heterologous (from other chromosomes) or alternative (within the BCL-6 locus) regulatory sequences may disrupt the gene's normal expression pattern. A BCL-6 transcript of normal size was detected by Northern blot analysis of DLCL cells carrying either normal or truncated BCL-6. Some of the truncations were in the 5' flanking sequences and would therefore not be expected to generate structurally abnormal transcripts.

Experimental Discussion:

Zinc-finger encoding genes are candidate oncogenes as they have been shown to participate in the control of cell proliferation, differentiation, and organ pattern formation (24). In fact, alterations of zinc-finger genes have been detected in a variety of tumor types. These genes include PLZF (32) and PML (35-38) in acute promyelocytic leukemia; EVI-1 (38-39) in mouse and human myeloid leukemia, TTG-1 (40) in T-cell CLL, HTRX (41-43) in acute mixed-lineage leukemia, and WT-1 (44) in Wilm's tumor. Terminal differentiation of hematopoietic cells is associated with the down-regulation of many Krüppel-type zinc-finger genes. Thus, constitutive expression of BCL-6, caused by chromosomal rearrangements, interferes with normal B-cell differentiation, thereby contributing to the abnormal lymph node architecture typifying DLCL.

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Given that DLCL accounts for ~80% of NHL mortality (7), the identification of a specific pathogenetic lesion has important clinicopathologic implications. Lesions in BCL-6 may help in identifying prognostically distinct subgroups of DLCL. In addition, since a therapeutic response can now be obtained in a substantial fraction of cases (7), a genetic marker specific for the malignant clone may be a critical tool for the monitoring of minimal residual disease and early diagnosis of relapse (45).

The gene cloned from chromosomal translocations affecting band 3q27, which are common in DLCL codes for a 79 kD protein that is homologous with zinc-finger transcription factors. In 33% (13/39) of DLCL samples, but no in other types of lymphoid malignancies, the BCL-6 gene is truncated within its 5' noncoding sequences, suggesting that its expression is deregulated. Thus, BCL-6 is a proto-oncogene specifically involved in the pathogenesis of DLCL.

REFERENCES FOR SECTION II:

1. Chaganti, R.S.K., et al. (1989) Molecular Diagnostics of Human Cancer, pp. 33-36.
2. Nathwani, B.N., (1992) Neoplastic Hemopathology, pp. 555-601.
3. Tsujimoto, Y., et al. (1985) Science 228:1440.
4. Cleary, M.L. and Sklar, J., (1985) Proc. Natl. Acad. Sci. U.S.A. 82:7439.
5. Bakshi, A., et al. (1985) Cell 41:899.
6. Korsmeyer, S.J., (1992) Blood 80:879.
7. Magrath, I.T., (1990) The Non-Hodgkin's Lymphomas pp. 1-14.

-48-

8. Dalla-Favera, R., et al. (1982) Proc. Natl. Acad. Sci. U.S.A. 79:7824.
9. Taub, R., et al. (1982) Proc. Natl. Acad. Sci. U.S.A. 79:7837.
10. Dalla-Favera, R., (1993) Causes and Consequences of Chromosomal Translocations pp. 313-321.
11. Tsujimoto, Y., et al. (1985) Nature 315:340.
12. Meeker, T.C., et al. (1989) Blood 74:1801.
13. Motokura, T., et al. (1991) Nature 350:512.
14. Williams, M.E., et al. (1991) Blood 78:493.
15. Raffeld, M., and Jaffe, E., (1991) Blood 78:259.
16. Ladanyi, M., et al. (1991) Blood 77:1057.
17. Offit, K., et al. (1989) Bri. J. Haematol. 72:178.
18. Offit, K., et al. (1989) Blood 74:1876.
19. Bastard, C., et al. (1992) Blood 79:2527.
20. Ye, B.H., et al. (1993) Cancer Res 53:2732.
21. Baron, B.W., et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:5262.
22. Feinbert, A.P., and Vogelstein, B., (1983) Anal. Biochem. 132:6.
23. Kozak, M., (1989) J. Cell. Biol 108:229.
24. El-Baradi, T., and Pieler, T., (1991) Mech. Dev

-49-

35:155.

25. Rosenbert, U.B., et al. (1989) Nature 319:336.

26. Bellefroid, E.J., et al. (1989) DNA 8:377.

27. Knochel, W., et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6097.

28. Bellefroid, E.J., et al. (1991) DNA 88:3608.

29. Harrison, S.D., and Travers, A.A., (1990) EMBO J. 9:207.

30. DiBello, R.R., et al. (1991) Genetics 129:385.

31. Chardin, P., et al. (1991) Nucleic Acid Res. 19:1431.

32. Chen, Z., et al. (1993) EMBO J. 12:1161.

33. Koonin, E.V., et al. (1992) Trends Biochem. Sci. 17:213.

34. Xue, F., and Cooley, L., (1993) Cell 72:681.

35. de Thé, H., et al. (1991) Cell 66:675.

36. Kazizuka, A., et al. (1991) Cell 66:663.

37. Pandolfi, P.P., et al. (1991) Oncogene 6:1285.

38. Morishita, K., et al. (1988) Cell 54:831.

39. Fichelson, S., et al. (1992) Leukemia 6:93.

40. McGuire, E.A., et al. (1989) Cell Biol. 9:2124.

-50-

41. Djabali, M., et al. (1992) Nature Genet. 2:113.
42. Tkachuk, D.C., et al. (1992) Cell 71:691.
- 5 43. Gu, Y., et al. (1992) Cell 71:701.
44. Haber, D.A., et al. (1990) Cell 61:1257.
- 10 45. Medeiros, L.J., et al. (1992) Neoplastic Hemopathology, pp. 263-298.

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EXPERIMENTAL DETAIL SECTION IIIIntroduction:

5 Non Hodgkin's lymphoma (NHL), the most frequent tumor
occurring in patients between the ages of 20 and 40,
includes several distinct clinico-pathologic subtypes,
among which diffuse lymphoma with a large cell component
10 (DLCL) is the most clinically relevant in terms of
morbidity and mortality (1). DLCL include intermediate-
grade lymphomas with pure diffuse large- (DLCL), or mixed
small- and large-cell (MX-D) histology, as well as high-
grade immunoblastic (IMB) lymphoma. These tumors can occur
15 "de novo", accounting for 30-40% of initial NHL diagnosis
and, in addition, can represent the final "transformation"
stage of follicular lymphomas (FL), small lymphocytic
lymphoma and chronic lymphocytic leukemia. Considered
together, "de novo" and "post-transformation" DLCL
account for up to 80% of NHL mortality (1).

20 During the past decade, abnormalities involving proto-
oncogenes and tumor suppressor genes have been identified
in association with distinct NHL subtypes (2). These
genetic lesions represent important steps in
25 lymphomagenesis as well as tumor-specific markers which
have been exploited for diagnostic and prognostic purposes
(3,4). Examples include alterations of the MYC oncogene in
Burkitt lymphoma (BL), and of the BCL-2 and BCL-1
oncogenes in FL and mantle-cell NHL, respectively. With
30 respect to DLCL, several molecular alterations have been
detected at variable frequency, but none has been
specifically or consistently associated with the disease
(2). In this invention the frequency and disease-
specificity of BCL-6 (5-10) rearrangements among the
35 principal categories of lymphoproliferative disease,
including different NHL subtypes, acute and chronic
lymphoid leukemias and multiple myeloma is demonstrated.

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Materials and Methods:

Samples of lymphnode biopsies, bone marrow aspirates and peripheral blood were collected by standard diagnostic procedures during the course of routine clinical evaluation in the Division of Surgical Pathology, Department of Pathology, Columbia University. In all instances, the specimens were collected before specific anti-tumor treatment. Diagnoses were based on the results of histopathologic, immunophenotypic and immunogenotypic analysis (11). In all cases, the fraction of malignant cells in the pathologic specimen was at least 70% as determined by cytofluorimetric or immunohistochemical analysis of cell-surface markers or antigen receptor (immunoglobulin heavy chain and T cell receptor β chain) gene rearrangement analysis (11).

Genomic DNA was prepared from diagnostic specimens by cell lysis, proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. For Southern blot analysis, 6 μ g of DNA were digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized and transferred to Duralose filters (Stratagene, La Jolla, CA). Filters were then hybridized with the BCL-6-specific Sac 4.0 probe (10) that had been 32 P-labelled by the random priming technique. After hybridization, filters were washed in 0.2X SSC (1X SSC = 0.15 M NaCl + 0.015 M sodium citrate / 0.5% sodium dodecyl sulfate) for 2 hours at 60°C and then subjected to autoradiography for 24-48 hours at -80°C using intensifying screens.

All NHL cases were also analyzed for rearrangement of the BCL-2 gene using the previously described probes corresponding to the MBR and MCR regions. Immunophenotypic analysis of immunoglobulin and cell surface marker expression was performed as previously described (11).

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Comparisons of histologic subsets with or without BCL-6 rearrangement were made utilizing the method of inferences from proportions (12).

5 Experimental Results:

10 The tumor panel (Table 2) used for this study is representative of the major categories of lymphoproliferative disease including NHL, 125 cases, ALL 45, CLL 51 and MM 23. The NHL series was representative of low- 41, intermediate- 45 and high-grade 24 subtypes according to the Working Formulation. Fifteen cases of cutaneous T-cell NHL were also included.

15 The presence of BCL-6 rearrangements was analyzed by Southern blot hybridization of tumor DNAs using a probe (Sac 4.0) (10) and restriction enzymes (BamHI and XbaI) which, in combination, explore a region of 15.2 Kb containing the 5' portion of the BCL-6 gene (first exon, 20 7.5 Kb of first intron and 7.4 Kb of 5' flanking sequences) (10). This region was previously shown to contain the cluster of breakpoints detected in NHL. No additional rearrangements were found using probes and restriction enzymes exploring approximately 10kb either 5' or 3' to BCL-6 sequences

25 The results of this analysis are summarized in Table 2 and representatively shown in Figures 13A-13B. All cases of ALL, CLL and MM showed a normal BCL-6 gene. Eighteen of 30 the 125 NHL cases displayed BCL-6 rearrangements. Among distinct NHL histologic subtypes, rearrangements were detected in 16/45 (35.5%) DLCL and in 2/31 (6.4%) FL ($p < .001$). One of these 2 FL cases showed both follicular and diffuse patterns of growth. Among DLCL, rearrangements 35 were significantly more frequent in DLCL (15/33, 45.4%) than in MX-D (1/10, 10%) ($p < .01$), suggesting that these genetic lesions may be specifically associated with the diffuse large cell component of these tumors. All of the

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DLLC cases displaying BCL-6 rearrangements lacked BCL-2 rearrangements which were found in only two 2 DLLC cases. Although cytogenetic data were not available for the panel of tumors studied, the frequency of BCL-6 rearrangements far exceeds that expected for 3q27 aberrations (10-12% in DLLC) (8, 9), suggesting that BCL-6 rearrangements can occur as a consequence of submicroscopic chromosomal aberrations.

In order to determine whether the presence of BCL-6 rearrangements correlated with distinct immunophenotypic features of DLLC, the entire panel was analyzed for expression of immunoglobulin κ and λ light chains, and B cell-associated antigens CD19, CD20 and CD22 (11). As expected, the expression of these markers was variable in the DLLC cases tested. However, no correlation with the BCL-6 rearrangement status was found.

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Table 2.**Rearrangements of the BCL-6 gene in lymphoid tumors**

	TUMOR	HISTOTYPE	REARRANGED/TESTED	%
5	<hr/>			
	<u>NHL</u>			
	Low grade:	SL	0/10	0
		SCC-F	2*/18	11
		MX-F	0/13	0
10	Intermediate grade:			
		MX-D	1/10	10
		DLCL	15/33	45
		SCC-D	0/2	0
15	High grade:			
		IMB	0/2	0
		SNCL	0/22	0
20	Others:	CTCL	0/15	0
	<u>ALL</u>			
		B-lineage:	0/34	0
		T-lineage:	0/11	0
25	<u>CLL</u>	B-lineage:	0/41	0
		T-lineage:	0/10	0
	<u>MM</u>		0/23	0
30	<hr/>			
35	NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; SL, small lymphocytic; SCC-F, follicular small cleaved cell; MX-F, follicular mixed; MX-D, diffuse mixed cell; DLCL, diffuse large cell; SCC-D, diffuse small cleaved cell; IMB, immunoblastic; SNCL, small non-cleaved cell lymphoma; CTCL, cutaneous T-cell lymphoma. *: one case showed follicular and diffuse growth patterns.			

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Experimental Discussion:

In this study, BCL-6 rearrangement is established as the most frequent abnormality detectable in DLLC. Previous studies have indicated MYC and BCL-2 rearrangements detectable in 5-20% and 20% of DLLC, respectively (13). Compared to those lesions, which are also commonly associated with Burkitt's lymphoma (MYC) and FL (BCL-2), BCL-6 rearrangements appear to be more disease-specific since they were exclusively found in DLLC with the exception of 2 of 45 FL cases. Considering that one of these two FL cases displayed areas of diffuse histology, it is conceivable that BCL-6 rearrangements may be occasionally associated with atypical FL cases with mixed follicular and diffuse components. The recurrent and specific association between DLLC and structural lesions of a gene coding for a zinc finger-type transcription factor related to several known proto-oncogenes 10 suggests that these abnormalities may play a role in pathogenesis of DLCL.

Among the heterogeneous DLLC spectrum, BCL-6 rearrangements were significantly more frequent in tumors displaying a pure diffuse large cell histology (DLCL) all of which lacked BCL-2 rearrangements. Considering that DLCL can originate both "de novo" and from the "transformation" of FL, and that the latter typically carry BCL-2 rearrangements, results suggest that BCL-6 rearrangements may be specifically involved in the pathogenesis of "de novo" DLLC. This conclusion is consistent with recent findings indicating that other genetic alterations, namely the inactivation of the p53 tumor suppressor gene, may be involved in the transformation of FL to DLLC (14).

The results presented herein have relevant diagnostic and prognostic implications. DLLC represent a heterogeneous group of neoplasms which are treated homogeneously despite the fact that only 50% of patients experience long-term

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disease free survival (1). The presence of a marker such as BCL-6 rearrangement identifies a sizable subset of cases with a distinct pathogenesis and, distinct biological behavior.

5 The pathogenesis of non-Hodgkin lymphoma with a large-cell component (DLCL, including diffuse large-cell, DLCL, diffuse mixed-cell, MX-D, and immunoblastic, IMB) is unknown. The incidence and disease-specificity of BCL-6
10 rearrangements in a large panel of lymphoid tumors, including acute and chronic lymphoid leukemias (96 cases), various NHL types (125 cases), and multiple myelomas (23 cases) has been tested. BCL-6 rearrangements were found in
15 16/45 (35.5%) DLCL, more frequently in DLCL (15/33, 45%) than in MX-D (1/10, 10%), in 2/31 (6.4%) follicular NHL, and in no other tumor types. BCL-6 rearrangements represent the first genetic lesion specifically and recurrently associated with DLCL and should prove useful for understanding the pathogenesis as well as for the
20 clinical monitoring of these tumors.

REFERENCES FOR SECTION III

1. Magrath, I.T. (1990) The Non-Hodgkin's Lymphomas: An
25 Introduction, The Non-Hodgkin's Lymphomas, Edward Arnold, London, p 1.
2. Gaidano, G., and Dalla-Favera, R. (1993) Biologic and
molecular characterization of non-Hodgkin's lymphoma,
30 Curr. Opin. Oncol. 5:776.
3. Gribben, J.G., et al. (1991) Immunologic purging of
marrow assessed by PCR before autologous bone marrow
transplantation for B-cell lymphoma, N. Engl. J. Med.
35 325:1525.
4. Yunis, J.J., et al. (1989) Bcl-2 and other genomic
alterations in the prognosis of large-cell lymphoma,

-58-

N. Engl. J. Med. 320:1047.

5. Ye, B.H., et al. (1993) Cloning of bcl-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma, Cancer Res. 53:2732.
5
6. Baron, B.W., et al. (1993) Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas, Proc. Natl. Acad. Sci. USA 90: 5262.
10
7. Kerckaert, J.P., et al. (1993) LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas, Nature Genet. 5:66.
15
8. Offit, K, et al. (1989) t(3;22)(q27;q11): A novel translocation associated with diffuse non-Hodgkin's lymphoma, Blood 74: 1876.
20
9. Bastard, C, et al. (1992) Translocations involving band 3q27 and Ig gene regions in non-Hodgkin's lymphoma, Blood 79: 2527.
25
10. Ye, B.H., et al. (1993) Alterations of a zinc-finger encoding gene, BCL-6, in diffuse large-cell lymphoma, Science 262:747.
- 30 11. Knowles, D.M. (ed.) (1992) Neoplastic Hemopathology, Williams & Wilkins, Baltimore, MD.
12. Armitage, P., (1977) Statistical methods in medical research, Blackwell Scientific Publications, London,
35 p. 11.
13. Chaganti, R.S.K., et al. (1989) Specific translocations in non-Hodgkin's lymphoma: incidence,

-59-

molecular detection, and histological and clinical correlations, Cancer Cells 7:33.

- 5 14. Lo Coco, F., et al. (1993) p53 mutations are associated with histologic transformation of follicular lymphoma, Blood 82:2289.

-60-

EXPERIMENTAL DETAIL SECTION IVIntroduction:

5 Non-Hodgkin lymphomas (NHL) represent one of the most
common malignancies associated with human immunodeficiency
virus (HIV) infection, and are recognized as an acquired
immunodeficiency syndrome (AIDS)-defining condition (1-3).
10 Since their initial observation in 1982 (4), the incidence
of AIDS-associated NHL (AIDS-NHL) has been consistently
increasing (1, 2), and they now represent the most frequent
HIV-associated malignancy in some AIDS risk groups, namely
the hemophiliacs (5). Indeed, some estimates project that
15 10 to 20% of all new NHL cases in the United States may
eventually be related to AIDS (6).

AIDS-NHL are almost invariably B-cell derived NHL (1, 2, 7-
12). When compared with NHL of similar histology arising
in the immunocompetent host, AIDS-NHL display distinctive
20 clinical features, including late stage at presentation,
poor prognosis, and the frequent involvement of extranodal
sites (1, 2, 7-12). Systemic AIDS-NHL are histologically
heterogeneous, and have been initially classified into
three distinct categories, including small non cleaved cell
25 lymphoma (SNCCCL), large non cleaved cell lymphoma (LNCCL),
and large cell-immunoblastic plasmacytoid lymphoma (LC-
IBPL) (7, 9). Subsequently, most investigators have agreed
to classify LNCCL and LC-IBPL as a single category under
the term of diffuse large cell lymphoma (DLCL).

30 Some progress has been made in elucidating the molecular
pathogenesis of AIDS-SNCCCL (1-3). AIDS-SNCCCL is associated
at variable frequency with multiple genetic lesions,
including Epstein Barr virus (EBV) infection, c-MYC
35 translocation, RAS gene family mutation, and p53
inactivation by point mutation and allelic loss (1, 3, 13-
25). On the other hand, the pathogenesis of AIDS-DLCL is
relatively less defined. EBV infection appears to be the

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only genetic lesion associated with a significant fraction of these tumors, particularly with the subset displaying plasmacytoid features, p53 lesions have not been found and c-MYC activation is restricted to a small minority of cases (1-3, 13-25).

Materials and Methods:

Pathologic samples. Biopsy samples of lymph node, bone marrow, peripheral blood, or other involved organs from forty patients with AIDS were collected during the course of standard diagnostic procedures. Thirty-two samples were derived from patients referred to the Department of Pathology, New York University, New York, NY or to the Department of Pathology, Columbia University, New York, NY. Eight samples were derived from patients referred to the Departments of Hematology and Pathology, University of Southern California School of Medicine, Los Angeles, CA. Diagnosis was based on analysis of histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of Immunoglobulin (Ig) gene rearrangement (32). In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorometric or tissue section immunohistochemical analysis of cell surface markers and by Ig gene rearrangement analysis.

DNA extraction and Southern blot analysis. DNA was purified by digestion with proteinase K, "salting out" extraction, and precipitation by ethanol (33). For Southern blot analysis (34), 6 μ g of DNA was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to Duralon filters (Stratagene, LA Jolla, CA), and hybridized to probes which had been 32 P-labeled by the random primer extension method (35). Filters were washed in 0.2 X SSC (NaCl/Na citrate)/0.5% sodium dodecyl sulphate (SDS) for 2 hours at 60°C and then autoradiographed using

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intensifying screens (Quanta III; Dupont, Boston, MA).

DNA probes. Immunoglobulin gene rearrangement analysis was performed using a J_H probe(36) (a gift of Dr. Korsmeyer) on *Hind*III, *Eco*RI, and *Bam*HI digests. The organization of the *BCL-6* locus was investigated by hybridization of *Xba*I, *Bam*HI, and *Bgl*II digested DNA to the human *BCL-6* probe Sac4.0 (26-27). In selected cases, a second probe representative of the *BCL-6* locus, Sac0.8, was also used. The organization of the c-MYC locus was analyzed by hybridization of *Eco*RI and *Hind*III digested DNA to the human c-MYC locus was analyzed by hybridization of *Eco*RI and *Hind*III digested DNA to the human c-MYC probe MC413RC, representative of the third exon of the c-MYC gene (37). The presence of the EBV genome was investigated with a probe specific for the EBV termini (5.2 Kb *Bam*HI-*Eco*RI fragment isolated from the fused *Bam*HI terminal fragment NJ-het) (38).

Experimental Results:

Forty cases of systemic AIDS-NHL were studied, including 13 SNCLL and 24 DLCL (8 LNCLL and 16 LC-IBPL). In addition, three cases of CD30+ lymphomas, which have been sporadically reported in AIDS (39), were also included. All cases displayed a predominant monoclonal B-cell population as determined by Ig gene rearrangement analysis.

Analysis of *BCL-6* rearrangements. The *BCL-6* gene contains at least 9 exons spanning approximately 26 Kb of genomic DNA (27). Sequence analysis has shown that the first exon is non-coding and that the translation initiation codon is located within the third exon (27). Rearrangements of *BCL-6* can be detected by Southern blot analysis using a probe (Sac4.0) and restriction enzymes (*Bam*HI and *Xba*I) which, in combination, explore a region of 15.2 Kb containing the 5' portion of the *BCL-6* gene (27) (Figures 14A-14C). This same region was previously shown to contain the cluster of

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chromosomal breakpoints detected in NHL of the immunocompetent host (27, 29). Cases showing an abnormally migrating band in only one digest were further studied by hybridizing the Sac4.0 probe to additional digests (*Bgl*II) or, alternatively, by hybridizing *Bam*HI and *Xba*I digests to a probe (Sac0.8) derived from the *BCL-6* first intron, which, being located 3' of the breakpoint cluster, explores the reciprocal chromosome 3 (Figures 14A-14C). Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.

Rearrangements of *BCL-6* were detected 5/24 AIDS-DLCL (20.8%), both in the LNCCL (2/8; 25%) and in the LC-IBPL (3/16; 18.7%) variants (Table 3 and Figures 14A-14C). All cases of AIDS-SNCCL and CD30+ lymphomas displayed a germline *BCL-6* locus. The location of the breakpoints detected in AIDS-HNL corresponds to the pattern most commonly observed in DLCL of the immunocompetent host.

Table 3.

Frequency of *BCL-6* rearrangements in AIDS-NHL

SNCCL ^a	DLCL ^b		CD30+NHL ^c
	LNCCL	LC-IBPL	
0/13	2/8	3/16	0/3

^a: SNCCL, small non cleaved cell lymphoma

^b: DLCL, diffuse large cell lymphoma. The DLCL included in the panel can be further distinguished into two subgroups (LNCCL, large non cleaved cell lymphoma; and LC-IBPL, large cell immunoblastic-plasmacytoid lymphoma) as previously reported (7,9).

^c: Non-Hodgkin lymphoma expressing the CD30 cell surface antigen (39).

Other genetic lesions. The other genetic lesions

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investigated in the panel of AIDS-NHL included infection by EBV of the tumor clone, activation of the c-MYC and RAS proto-oncogenes, and inactivation of the p53 tumor suppressor gene. The experimental strategies used to investigate these lesions have been described in detail elsewhere (13, 45, 40). For some of the

cases, the molecular characterization of these genetic lesions have been previously reported (13, 14, 41); for the other cases, it has been assessed in the course of this study.

EBV infection was assessed by Southern blot hybridization using a probe representative of the EBV termini (38) which allows to analyze clonality in EBV-infected tissues (23) (Figures 16A-16C). A monoclonal infection was detected in 5/13 (38%) SNCLL, 17/24 DLCL (71%) [3/8 (37.5%) LNCLL and 14/16 (87.5%) LC-IBPL], and 3/3 (100%) CD30+ cases.

Rearrangements of c-MYC were tested by hybridizing *HindIII* and *EcoRI* digested DNAs with a probe representative of c-MYC exon 3(41) (Figures 16A-16C). Rearrangements were present in 13/13 SNCLL (100%), 5/24 (20.8%) DLCL [2/8 (25%) LNCLL and 3/16 (18.7%) LC-IBPL], and 2/3 CD30+ cases.

Mutations of p53 and RAS were analyzed by a two step strategy. Single strand conformation polymorphism (SSCP) analysis was applied to p53 exons 5 through 9 (in 29 cases) or p53 exons 5 through 8 (in 6 cases) and to N-, K-, and H-RAS exons 1 and 2 (in 29 cases); cases displaying an altered electrophoretic pattern by SSCP were further studied by DNA direct sequencing of the PCR product. p53 mutations were scored in 8/13 (61.5%) SNCLL, but in none of the DLCL tested (0/22). Finally, RAS activation by point mutation was positive in 3/13 (23%) SNCLL and in 1/16 (6%) DLCL tested.

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The molecular features of the cases displaying *BCL-6* rearrangements are listed in Table 4. Overall, *BCL-6* rearrangements were detected both in the presence and in the absence of clonal EBV infection of the tumor, whereas
5 *c-MYC* alterations and *p53* mutations were consistently absent in the cases displaying *BCL-6* rearrangements.

Experimental Discussion:

Diffuse large cell lymphoma (DLCL) represents the most frequent type of AIDS-NHL in the HIV-infected adult (8). Despite its epidemiologic relevance, the molecular pathogenesis of these tumors is largely unclarified (3). Analysis of the genomic configuration of *BCL-6* in a panel of AIDS-NHL indicates that *BCL-6* rearrangements are involved in approximately 20% of AIDS-DLCL, whereas they are consistently negative in AIDS-SNCCL. In this respect, *BCL-6* rearrangements may be considered the first identified genetic lesion specific for the DLCL type among AIDS-NHL. *BCL-6* rearrangements are present in both subgroups of DLCL, i.e. LNCCL and LC-IBPL, and occur both in the absence and in the presence of EBV infection of the tumor clone (Table 4). On the other hand, *BCL-6* rearrangements were never detected in AIDS-DLCL carrying *c-MYC* alterations (Table 4).

The molecular pathway leading to AIDS-SNCCL involves *c-MYC* rearrangements, *p53* mutations, and EBV infection in 100%, 60%, and 40% of the cases, respectively (13-26). The presence of somatic hypermutation in the immunoglobulin variable regions utilized by AIDS-SNCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors. The second genetic pathway is associated with AIDS-DLCL, involves EBV in the large majority of cases, as well as *c-MYC* and/or *BCL-6* rearrangements in a fraction of cases (13-26). These distinct pathogenetic mechanisms correlate with a number of clinical features which distinguish AIDS-SNCCL from AIDS-DLCL, including different age of onset and different CD4 counts at the time of lymphoma development (1,2,8).

Results suggest that the frequency of *BCL-6* rearrangements in AIDS-DLCL is significantly lower than that in DLCL in the immunocompetent host, where *BCL-6* rearrangements occur in more than 40% of the cases. It is possible that the

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genetic pathogenesis of these two groups of tumors is different, and that the molecular mechanisms active in AIDS-DLCL are characterized by a higher degree of heterogeneity. Among DLCL in the immunocompetent host, *BCL-6* rearrangements are associated with distinct clinical features, including the extranodal origin of the lymphoma and the lack of bone marrow involvement. In addition, the presence of this rearrangement appears to represent a favorable prognostic marker.

REFERENCES FOR SECTION IV:

1. Karp, J.E., and Broder, S. (1991) Acquired Immunodeficiency Syndrome and non-Hodgkin's lymphomas, Cancer Res. 51:4743.
2. Levine, A.M. (1992) Acquired Immunodeficiency Syndrome-related lymphoma, Blood 80:8.
3. Gaidano, G., and Dalla-Favera, R. (1992) Biologic aspects of human immunodeficiency virus-related lymphoma, Curr. Opinion Oncol. 4:900.
4. Ziegler, J.L., et al. (1982) Outbreak of Burkitt's like lymphoma in homosexual men, Lancet 2:631.
5. Ragni, M.V., et al. (1993) Acquired immunodeficiency syndrome-associated non-Hodgkin's lymphomas and other malignancies in patients with hemophilia, Blood 81:1889.
6. Gail, M.H., et al. (1991) Projection of the incidence of non-Hodgkin's lymphoma related to acquired immunodeficiency syndrome, J. Natl. Cancer Inst. 83:965.
7. Raphael, B.G., and Knowles, D.M. (1990) Acquired immunodeficiency syndrome-associated lymphomas,

-69-

Sermin. Oncol. 17:361.

8. Beral, V., et al. (1991) AIDS-associated non-Hodgkin lymphoma, Lancet 337:805.
- 5 9. Knowles, D.M., et al. (1988) Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS), Ann. Int. Med. 108:744.
- 10 10. Levine, A.M., et al. (1984) Development of B-cell lymphoma in homosexual men, Ann. Intern. Med. 100:7.
- 15 11. Carbone, A., et al. (1991) A clinicopathologic study of lymphoid neoplasias associated with human immunodeficiency virus infection in Italy, Cancer 68:842.
- 20 12. Ioachim, H.L., et al. (1991) Acquired immunodeficiency syndrome-associated lymphomas: Clinical, pathologic, immunologic, and viral characteristics of 111 cases, Hum. Pathol. 22:659.
- 25 13. Ballerini, P., et al. (1993) Multiple genetic lesions in acquired immunodeficiency syndrome-related non-Hodgkin's lymphoma, Blood 81:166.
- 30 14. Gaidano, G., et al. (1993) In vitro establishment of AIDS-related lymphoma cell lines: phenotypic characterization, oncogene and tumor suppressor gene lesions, and heterogeneity in Epstein-Barr virus infection, Leukemia 7:1621.
- 35 15. Groopman, J.E., et al. (1986) Pathogenesis of B-cell lymphoma in a patient with AIDS, Blood 67:612.
16. Pelicci, P.-G., et al. (1986) Multiple monoclonal B cell expansions and c-myc oncogene rearrangements in acquired immune deficiency syndrome-related

-70-

lymphoproliferative disorders. Implications for lymphomagenesis, J. Exp. Med. 164:2049.

- 5 17. Subar, M., et al. (1988) Frequent c-myc oncogene activation and infrequent presence of Epstein-Barr Virus genome in AIDS-associated lymphoma, Blood 72:667.
- 10 18. Haluska, F.G., et al. (1989) Molecular resemblance of an AIDS-associated lymphoma and endemic Burkitt lymphomas: implications for their pathogenesis, Proc. Natl. Acad. Sci. USA 86:8907.
- 15 19. Meeker, T.C., et al. (1991) Evidence for molecular subtypes of HIV-associated lymphoma: division into peripheral monoclonal, polyclonal and central nervous system lymphoma, AIDS 5:669.
- 20 20. Epstein-Barr virus and AIDS associated lymphomas. Editorial, Lancet 338:979, (1991).
- 25 21. Hamilton-Dutoit, S.J., et al. (1991) Detection of Epstein-Barr virus genomes in AIDS related lymphomas: sensitivity and specificity of in situ hybridization compared with Southern blotting, J. Clin. Pathol. 44:676.
- 30 22. Hamilton-Dutoit, S.J., et al. (1991) AIDS-related lymphoma. Histopathology, immunophenotype, and association with Epstein-Barr virus as demonstrated by in situ nucleic acid hybridization, Am. J. Pathol. 138:149.
- 35 23. Neri, A., et al. (1991) Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma, Blood 77:1092.

-71-

24. Nakamura, H., et al. (1993) Mutation and protein expression of p53 in acquired immunodeficiency syndrome-related lymphomas, Blood 82:920.
- 5 25. Carbone, A., et al. (1993) Human immunodeficiency virus-associated systemic lymphomas may be subdivided into two main groups according to Epstein-Barr viral latent gene expression, J. Clin. Oncol. 1:1674.
- 10 26. Ye, B.H., et al. (1993) Cloning of BCL-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma, Cancer Res. 53:2732.
- 15 27. Ye, B.H., et al. (1993) Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large cell-lymphoma, Science 262:747.
- 20 28. Baron, B.W., et al. (1993) Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas, Proc. Natl. Acad. Sci. USA 90:5262.
- 25 29. Kerckaert, J.-P., et al. (1993) LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphoma, Nature Genet. 5:66.
- 30 30. Deweindt, C., et al. (1993) Cloning of a breakpoint cluster region at band 3q27 involved in human non-Hodgkin's lymphoma, Genes, Chrom. & Cancer 8:149.
- 35 31. Bastard, C., and Tilly, H. (1993) Response to letter "t(2;3)(p12;q27) in Hodgkin's disease of a human immunodeficiency-virus positive patient with hemophilia", by Schlegelberger B, Grote W, Wacker HH, Bartels H., Blood 81:265.

-72-

32. Knowles, D.M., et al. (1986) T-cell receptor Beta chain (T_B) gene rearrangements: genetic markers of T-cell lineage and clonality, Hum. Pathol. 17:546.
- 5 33. Miller, S.A., et al. (1988) A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acid Res. 16:1215.
- 10 34. Sambrook, J., et al. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.
- 15 35. Feinberg, A.P., and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, Anal. Biochem. 132:6.
- 20 36. Korsmeyer, S.J., et al. (1981) Developmental hierarchy of immunoglobulin gene rearrangement in human leukemic pre-B cells, Proc. Natl. Acad. Sci. USA 78:7096.
- 25 37. Dalla-Favera, R., et al. (1981) Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells, Proc. Natl. Acad. Sci. USA 78:7096.
38. Weiss, L.M., et al. (1987) Epstein-Barr virus DNA in tissues of Hodgkin's disease, Am. J. Pathol. 129:86.
- 30 39. Chadburn, A., et al. (1993) CD30(Ki-1) positive anaplastic large cell lymphomas in individuals infected with the human immunodeficiency virus, Cancer 72:3078.
- 35 40. Gaidano, G., et al. (1991) p53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukemia, Proc. Natl. Acad. Sci. USA 88:5413.

-73-

41. Shibata, D., et al. (1993) Epstein-Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus, Blood 81:2102.

EXPERIMENTAL DETAIL SECTION VIntroduction:

5 The group of diffuse lymphomas with a large cell component (DLCL), including diffuse mixed, immunoblastic, and large cell subtypes, and the group of follicular lymphomas, each comprise about 40 per cent of non-Hodgkin's lymphomas (NHL) in this country (1). Together, the incidence of NHL is
10 increasing at 3 to 4 per cent a year, a rate second only to that of malignant melanoma and lung cancer in women (2). Despite significant advances in treatment, approximately half of patients with DLCL will succumb to their disease, although "high risk" individuals may successfully be
15 treated by intensive chemotherapy and radiotherapy regimens including autologous bone marrow transplantation (3-7). The formulation of prognostic models allow clinical trials to be directed toward groups of patients with different risks for failure after conventional treatment (5).

20 Cytogenetic studies as well as molecular genetic analysis of alterations involving proto-oncogenes and tumor suppressor genes have provided insights into the pathogenesis of NHL, and have also contributed diagnostic
25 and prognostic markers (8,9). Examples include rearrangements of the BCL-2 gene at 18q21 observed in up to 85 per cent of follicular lymphomas, the BCL-1 gene at 11q13 rearranged in intermediate differentiation NHL, and the MYC gene, perturbed in Burkitt's lymphoma (8,9). While
30 no recurring genetic abnormality has been specifically associated with diffuse large cell lymphoma, rearrangement of BCL-2 has been observed in 20 to 30 per cent of cases, where it has been associated with decreased overall or disease free survival (10-12). Chromosomal translocations
35 including those involving the MYC proto-oncogene, while noted in DLCL, were not as prognostically significant as other recurring chromosomal abnormalities (8,13).

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BCL-6 (14-19) rearrangement is found to denote a subset of DLLC characterized by extranodal presentation and a favorable clinical outcome. These results indicate that, in concert with other clinical features, this molecular marker may be utilized as a prognostic indicator at the time of diagnosis.

Materials and Methods:

This study was comprised of 102 cases of DLLC studied at diagnosis with documented clonal rearrangement of the IGH gene and DNA available for further analysis, derived from 229 DLLC serially ascertained over a nine year period. Excluded were 127 cases studied at relapse, T cell DLLC, or cases for which no DNA was available. For this study, DLLC was defined as lymphomas of diffuse large cleaved, non-cleaved, immunoblastic, or mixed subtype, according to the International Working Formulation (20) as classified by a hematopathologist (DCS or DF). Cytogenetic analysis was attempted on each of the specimens as previously described (21). For detection of BCL-6 rearrangements, DNA from each case was digested with BamHI and XbaI and subjected to Southern blot analysis utilizing a 4kb SacI-SacI fragment of the BCL-6 gene as a probe (19). Cases which did not yield metaphases for karyotypic analysis were also analyzed for rearrangement of the MBR and MCR breakpoint regions of the BCL-2 gene, as previously described (11). Aggregate descriptions of 47 of the cases in the current series were included in prior reports of cytogenetic abnormalities in DLLC (11, 13, 14). A detailed molecular analysis of 8 cases (nos. 352, 755, 1098, 1254, 1403, 1444, 1445) demonstrating BCL-6 rearrangement has been reported separately (19).

For each case, clinical data were compiled as previously described (22). Stage was assessed according to the modified Ann Arbor criteria (25). For the purposes of separate evaluation of number of extranodal sites of

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disease, radiographs or pathologic involvement of these sites were scored. In the quantitation of extranodal sites of disease as a prognostic variable, bone marrow, but not splenic involvement was scored, in accord with the International Prognostic Index (5).

Clinical endpoints including complete response and freedom from progression were defined as previously described (3). Of 102 patients with DLCL genetically analyzed prior to cytotoxic treatments, 93 received systemic chemotherapy. Nine patients with early stage disease were treated by surgical resection and/or radiation therapy. All patients were treated with curative intent. Chemotherapy treatments were classified into three groups: NHL-4, CHOP and BACOP (1st generation); m-BACOD, NHL-7 (2nd generation); MCOP_B, NHL-9, NHL-14, NHL-15, L-20 (3rd generation) (4,24,29). Eight patients expired before completion of therapy, with incomplete staging evaluations, or of infectious complications during or shortly after treatment. These cases were considered not valuable for the determination of remission status, but were included in the analysis of overall survival and freedom from progression. One patient was judged to be a complete remission which was confirmed by autopsy after expiration due to infectious complications 3 weeks after completion of protocol treatment. All deaths, regardless of cause were considered as endpoints in the analysis of overall survival. Median survival was determined by the method of Kaplan and Meler (30). Analysis of correlations between gene rearrangements and clinical features were performed utilizing Fisher's exact test (13). Means were compared utilizing two sample 't'-tests. Univariate comparisons of survival and duration free from progression were made by log rank test. Survival and freedom from progression estimates are quoted with confidence intervals (CI) given in parentheses. Multivariate analysis was performed utilizing the Cox regression model (31). Stepwise multiple logistic regression was used in the multivariate analysis of factors

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prognostic for achieving a complete response. For all statistical analyses, a $P < 0.05$ based on a 2-sided test was considered significant.

5 Experimental Results:

Of 102 cases of DLCL studied at diagnosis, 23 demonstrated BCL-6 rearrangement, 21 demonstrated t(14;18) or rearrangement of BCL-2, and 58 demonstrated no evidence of
10 either BCL-6 or BCL-2 rearrangement. Representative results of hybridization analysis for rearrangement of BCL-6 are depicted in Figures 17A-17B. The clinical characteristics of groups according to BCL-6 or BCL-2 rearrangement are summarized in Table 5. The histologic
15 subtypes and clinical features of the BCL-6 rearranged cases are shown in Table 6.

The key to Table 6 is as follows: Underlining signifies site from which biopsy was performed. Histology: DLCL = diffuse large cell; IMB - immunoblastic; DML = diffuse mixed lymphoma; LDH = lactate dehydrogenase in units/ml; (B) = bulky disease (> 8 cm or $1/3$ thoracic diameter); CHOP = cyclophosphamide, daunorubicin, vincristine, prednisone; MACOPB = methotrexate, daunorubicin, cyclophosphamide,
20 vincristine, prednisone, bleomycin; MBACOD = same drugs as MACOPB with dexamethasone instead of prednisone and drugs in different schedule; PrCyBom - drugs of MACOPB plus cytosine arabinoside, etoposide, methotrexate; L-20 - vincristine, cyclophosphamide, methotrexate, daunorubicin,
25 prednisone, cytosine arabinoside, 1-asparaginase, BCNU, 6-mercaptopurine, dactinomycin; L-20 includes randomization to autologous transplantation; NHL-7 = CHOP plus methyl GAG, etoposide(36), NHL-14 = short course PrCyBom; NHL-15 = high dose daunorubicin, vincristine,
30 cyclophosphamide(29); RT = radiation therapy; SURG = surgery CR = complete response; sCR = surgical complete response (all evaluable disease resected); PR = partial response; "+" = alive at last follow-up; e = expired; rel

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= relapse; NE = not evaluable; * = Patient 1445 had a history of low grade NHL of eyelid 7 years earlier, treated by radiotherapy. # Skin involvement of patient 252 was not noted in a prior report (14) and patient 1445 had a history of low grade NHL of eyelid 7 years earlier, treated by radiotherapy.

While each of the BCL-6 rearranged cases was classified as a DLCL, the range of morphologies included diffuse large cell (cleaved and non-cleaved), and less frequently, immunoblastic, or mixed histologies. Extensive necrosis and extranodal extension were common histologic features, and were present in one of two cases of BCL-6 rearrangement which did not show clinical evidence of extranodal disease.

The BCL-6 rearranged cases had a mean age of 64.1 years at presentation and a high frequency of extranodal involvement by disease; 19 of 23 cases had stages IE, IIE, IIIE or stage IV disease, compared to 48 of 79 of BCL-6 germline cases ($p=0.07$). Extranodal sites included muscle or subcutaneous tissues (6 cases), stomach (5 cases), lung or pleura (5 cases), skin, breast, bowel, thyroid, pancreas, or kidney, as assessed by biopsy or radiographic abnormalities which improved after chemotherapy. Of the 7 cases with stage IE or IIE disease, 5 were primary extranodal lymphomas, while 2 were extranodal extensions from a primary nodal site. Two cases were primary splenic lymphomas. In two cases, there was only peripheral adenopathy. Compared to BCL-6 germline cases, there was no significant difference in the proportion of BCL-6 rearranged cases with stage IV disease. Bone marrow involvement was observed in 15 of 75 BCL-6 germline cases biopsied, compared to only 1 of the 23 stage IV BCL-6 rearranged cases ($P=0.1$).

All but one of the 23 patients with BCL-6 rearrangement at the time of diagnosis received anthracycline-containing chemotherapy. This patient remained free of disease eight

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years after resection of a primary splenic large cell lymphoma. At median follow-up in excess of two years, 21 of the 23 patients with BCL-6 rearrangement survived; the actuarial survival was 91 per cent (CI 80 per cent to 100 per cent). Two patients expired during or immediately following treatment; an autopsy in one case revealed no evidence of lymphoma. This patient, and 19 others were judged to have achieved a complete remission after treatment. Two patients relapsed with recurrent disease in the lung and two patients had persistent subcutaneous masses. One of the relapse patients (case 295) went on to autologous transplanation and remains free of disease 78 months post-transplant.

With respect to known prognostic variables, the proportion of the BCL-6 rearranged cohort with LDH > 500 U per liter was similar to the proportion of the BCL-6 germline DLLC (3/23 versus 13/79; P=0.99). Five of 23 cases of DLLC with BCL-6 rearrangement demonstrated bulky disease, compared to 35 of 79 cases without BCL-6 rearrangement (P=0.1). The proportion of cases with "limited stage" (I, IE, II, or IIE) disease was comparable in the cohorts with and without BCL-6 rearrangement (Table 5).

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Table 5.**Characteristics of 102 cases of DLCL**

	BCL-6+	BCL-6- BCL2-	BCL-6+ BCL2+
5			
n=	23	58	21
Mean age (years)	64.1	52.7	62.8
10			
Mean lactate dehydrogenase (U/ml)	405	331	389
Mean extranodal sites	1.6	.93	.81
15			
Bone marrow involvement	1/23	8/54	7/21
20			
<u>Stage</u> I (IE)	3 (1)	3 (3)	0
II (IIE)	7 (6)	22 (6)	5
III (IIIE)	2 (1)	8 (2)	4
25			
IV	11	25	12
30			
<u>Histology</u> Diffuse large cell	20	53	19
Diffuse mixed	1	2	1
Immunoblastic	2	3	1
35			
<u>Treatment</u> 1st generation chemo.	12	16	10
2nd generation chemo.	1	10	3
3rd generation chemo.	9	24	8
other	1	8	0
40			
Complete Remission	20/23	35/50	15/21
Rate	86%	70%	71%
Projected survival at 36 months	91% (CI	59% (CI	46% (CI
45	80%-100%)	44%-74%)	21%-72%)
50			
Projected freedom from progression at 36 months	82% (CI 66%-98%)	56% (CI 43%-70%)	31% (CI 8%-53%)

Table 6.

Clinical features of 23 cases of DLCLC with BCL6 rearrangement.

Case Number	Age/ Sex	Stage	Extranodal Site	Histology	LDH (Bulk)	Treatment	Clinical Outcome
102	66/F	IIS	<u>spleen</u>	DLC	3624	SURG, CHOP	CR, 96+
147	61/F	IS	<u>spleen</u>	DLC	365 (B)	SURG, RT	sCR, 101+
252	54/M	IV	<u>spleen, skin#</u>	DLC	126	MACOPB	CR, 88+
278	68/M	IV	<u>pleura, iliac mass</u>	DLC	235 (B)	MACOPB	PR, 6e
295	46/M	IV	<u>lung</u>	DLC	179	MACOPB, L-20	CR, rel, 97+
352	53/F	IV	<u>stomach, liver, spleen, small bowel, pleural effusion</u>	IMB	775 (B)	MACOPB	CR, 81+
470	74/F	IV	<u>lung</u>	DLC	224	CHOP	CR, 30+
534	70/F	IIES	<u>spleen, mass involving pancreas</u>	DLC	278 (B)	CHOP	CR, 80+
763	79/F	IIE	<u>stomach</u>	DLC	196	CHOP	CR, 60+

970	75/M	IV	kidney, stomach	DLC	240	NHL-14	CR, 4e
1020	60/M	IIIE	tonsil, pancreas	DLC	303	CHOP	CR, 100+
1056	63/M	IIIE	<u>stomach</u>	DLC	213	SURG, MBACOD	CR, 100+
1058	59/M	IIIE	<u>axillary mass</u> <u>involving breast</u>	DLC	206	PrCyBom	CR, 37+
1098	74/F	IV	<u>subcutaneous</u> <u>masses</u>	DML	181	RT, CHOP	PR, 36+
1189	71/M	IV	<u>subcutaneous</u> <u>masses</u>	DLC	330	CHOP	PR, 21+
1254	74/F	IIIE	<u>thyroid</u>	DLC	196	CHOP/RT	CR, 27+
1264	76/F	IV	<u>Lung, liver</u> <u>spleen, kidney</u>	DLC	234	CHOP	CR, rel 27+
1299	50/M	IE	<u>deltoid mass,</u> <u>bone</u>	DLC	529 (B)	PrCyBom	CR, 16+
1363	47/M	III	NONE	DLC	129	NHL-15	CR, 16+
1403	62/M	I	NONE	IMB	222	CHOP, RT	CR, 11+

1407	71/M	IIE	<u>stomach</u>	DLC	206	SURG, CHOP	CR, 12+
1444	70/F	IV	<u>lung</u>	DLC	150	CHOP	CR, 14+
445	63/F	IV	neck mass involving muscle*, <u>bone</u> <u>marrow</u>	DLC	174	NHL-15	CR, 8+

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Multivariate analysts of clinical outcome. The median duration free from progression of disease was not reached in the BCL-6 rearranged DLLC compared to 70 months for BCL-6 germline cases, regardless of BCL-2 status ($P=0.009$) (Figure 18A). Projected freedom from progression at 36 months was 82% (CI 66%- 98%) and 49% (CI 37%-60%), respectively. Multivariate analysis revealed that four variables, BCL-6 status, stage IV disease, bulk of disease, and LDH (log transformed) were the most powerful prognostic indicators for freedom from progression (Table 7). Multivariate analysis of survival demonstrated that bulk, LDH, BCL-6 status, and stage IV disease were the most useful predictors of overall survival ($P=0.01$, $P=0.02$, $P=0.02$, $P=0.05$, respectively).

Table 7**Multivariate analysis of freedom from progression**

Variables selected into Cox regression model	Relative Risk	P value (Wald chi square)
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BCL-6 rearranged	0.18 (CI .04-.78)	0.007
Bulky disease	2.4 (CI 1.3-7.4)	0.01
Stage IV disease	2.1 (CI 0.98-5.2)	0.03
LDH (log transformed)	1.6 (CI 1.1-3.9)	0.05

The prognostic value of BCL-6 gene status was compared to risk variables calculated according to the International Prognostic Index⁵, including serum LDH level, stage, performance status, and number of extranodal sites. A cox regression analysis confirmed the independent prognostic value of BCL-6 gene status; patients with BCL-6 rearrangement had a relative risk (RR) of dying of .09 (CI

-85-

.02 to .42) compared to patients without BCL-6 rearrangement, controlling for the other prognostic variables in the model ($P=0.002$).

5 When cases were considered with respect to BCL-2 status, the BCL-2 rearranged cases demonstrated a trend for a decreased survival compared to BCL-2 germline cases, regardless of BCL-6 status ($P=0.12$). When BCL-6 and BCL-2 status were considered together (Figure 18B), BCL-6
10 rearranged cases demonstrated a projected actuarial survival at 36 months of 91% (CI 80%-100%) compared to 59% (CI 44%-74%) for the BCL-6 germline/BCL-2 germline cohort, and 46% (CI 21%-72%) for the BCL-2 rearranged cohort. While the logrank test between these three cohorts
15 demonstrated a difference in survival ($P=0.02$, Figure 18B), the major factor driving the significant summary P value was the better survival of the BCL-6 rearranged cohort. The projected freedom from progression at 36 months was 82% (CI 66%-98%), 56% (CI 43%-70%) and 31% (CI 8%-53%) for the
20 three groups. Median follow-up for survivors was two years. BCL-2 rearrangement did not emerge as an independent prognostic marker in the multivariate analysis of survival or freedom from progression.

25 There was also no prognostically significant effect of generation of chemotherapy treatment on survival, or freedom from progression ($P=0.95$, 0.21 , respectively). There was a trend for a higher complete response rate among the BCL-6 rearranged cohort (Table 5, $P=0.1$), although
30 logistic regression revealed that only the clinical parameters LDH, stage IV, and bulk of disease were independent predictors of response.

Relationship between BCL-6, BCL-2, and 8q24 rearrangements.

35 Of the 79 cases which lacked BCL-6 rearrangement, 21 demonstrated t(14;18)(q32;q21) or rearrangement of BCL-2 by molecular analysis. These cases were characterized by an older age at diagnosis, but were similar to the larger

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cohort of BCL-2 negative, BCL-6 negative cases with respect to LDH, and distribution of histologies (Table 6).

5 Nine cases of DLLC demonstrated t(8;14)(q24;q32). Three of these biopsies were from extranodal sites including liver, bone and soft tissue. Two additional cases were splenic lymphomas. In two cases, t(8;14) bearing DLLC also demonstrated BCL-6 rearrangement. There was no impact on survival of the t(8;14) in DLLC with or without BCL-6
10 rearrangement. The two cases of t(8;14) with co-incident BCL-6 rearrangement did not show evidence of histologic transformation or other unusual histologic features. One of these cases (no. 147) was the single case treated by splenectomy and radiation therapy alone. The second case
15 was the single BCL-6 rearranged case successfully salvaged by autologous transplantation.

Cytogenetic features, including the relationship between 3q27 and BCL-6 rearrangement. Of the 65 DLLC with
20 karyotypic abnormalities, 14 demonstrated translocations and one a deletion affecting band 3q27; only 11 among these 15 cases showed rearrangement of BCL-6. Five cases with apparently normal chromosomes 3 demonstrated BCL-6
25 rearrangements by DNA analysis.

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Experimental Discussion:

As a group, DLCL are among the most common forms of NHL seen in this country (1). These tumors have not, however, been associated with a characteristic genetic abnormality (8). Seen in the vast majority of follicular lymphomas, t(14;18)(q32;q21) or its molecular equivalent, BCL-2 rearrangement, have been observed in 20 to 30 per cent of DLCL (8). In such cases, the t(14;18) may reflect a follicular origin of these tumors. The recognition of translocations involving 3q27 and the sites of IG genes, 14q32, 22q11, and 2p12, in predominantly diffuse NHL led to the molecular cloning of BCL-6 (14-19). While not unique to diffuse large cell lymphomas, translocations affecting 3q27 were observed only in 7 of > 200 cases of follicular NHL with abnormal karyotype reported in catalog of chromosome abnormalities in cancer (32). Of 28 cases of follicular NHL analyzed in a prior study, none demonstrated rearrangement of BCL-6 (19). BCL-6 rearrangement was established as the most common genetic lesion specific to DLCL at the time of diagnosis.

Unlike 18q21 translocations in NHL which, to date only have involved IG gene loci as reciprocal partners, 3q27 translocations demonstrated a marked promiscuity of rearrangement partners. In addition to the sites of the IG genes, reciprocal translocations involving the 3q27-29 region with at least 12 other loci; a total of 79 DLCL with 3q27 translocations has been demonstrated.

Since 4 tumors in the current series with documented 3q27 aberrations did not reveal BCL-6 rearrangement with the probe used in this study, the true frequency of BCL-6 rearrangement in DLCL at diagnosis may be higher than the 23 per cent rate reported here. Additional breakpoints may be documented outside the recognized break cluster region of BCL-6 (19), in neighboring genes such as EV-1 (34), or in other genes not yet described. Such molecular

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heterogeneity is not unique in NHL; seemingly identical chromosomal translocations have been shown to demonstrate a diversity of breakpoints possibly involving different genes (35).

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The frequent occurrence of BCL-6 rearrangement in DLCLC characterized by extranodal involvement represents one of the few genetic markers for this subset of lymphoma (8). Rearrangements of BCL-1, BCL-2, or BCL-3 have been documented infrequently in extranodal lymphomas (36-38), while 5 of 12 gastric lymphomas in one series demonstrated MYC (8q24) rearrangement (38). The current series did not confirm the association between 8q24 rearrangement and gastric lymphoma, although t(8;14) was seen in five cases of extranodal lymphoma, one of which also showed BCL-6 rearrangement. The proportion of BCL-6 rearranged cases with stages IE, IIE, IIIE, or IV disease was higher than the proportion of BCL-6 germline DLCLC; in the latter group, stage IV disease was more commonly due to bone marrow involvement. Whether this association with extranodal involvement of disease reflects an effect of the primary deregulation of BCL-6 or "secondary" genetic events associated with tumor progression (8,21) is unclear. The observation of t(3;22), t(2;3), or t(3;14) as solitary cytogenetic abnormalities in some tumors (14,15), is consistent with a primary pathogenetic role for this translocation.

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While this analysis and two other reports did not confirm the very short survival of BCL-2 rearranged DLCLC initially reported (10, 12, 13, 39), the BCL-2 rearranged DLCLC did demonstrate a trend for a poorer overall survival. The finding of a favorable prognosis for the subset of stage IE-IIE extranodal DLCLC with BCL-6 rearrangement is consistent with prior reports of a good prognosis associated with localized extranodal large cell NHL treated with surgery or radiotherapy (40). Extranodal involvement in advanced stage disease, noted in the majority of the

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BCL-6 rearranged cases, has generally been considered a poor prognostic factor in large series of DLCL, although the negative impact of this feature was most evident when combined with other adverse indicators such as bulk, high LDH, or low performance status (5, 22, 41). In contrast, bone marrow involvement, observed in 22 percent of DLCL, and considered an extranodal site in the International Prognostic Index (5), was rare in the BCL-6 rearranged cohort. The favorable treatment outcome of the BCL-6 cohort, must also be tempered by the observation of relapse or residual disease in 3 of the patients still alive. An additional relapse case remains in remission 6 years after "salvage" autologous transplantation.

The BCL-6 rearranged cohort of DLCL also possessed other clinical markers of favorable prognosis; although comparable with respect to LDH and proportion with stage I-III disease, the proportion of cases with bulky disease or bone marrow involvement was lower in the BCL-6 rearranged cohort. Multivariate analysis suggested, however, that BCL-6 gene rearrangement added independent prognostic power when analyzed together with clinically-derived variables of the International Prognostic Index (5). This observation is illustrated by case 352, which displayed both BCL-6 rearrangement as well as clinical features consistent with a high level of risk in the International Index (elevated LDH, extensive extranodal involvement, low performance status, and stage IV disease), but who attained a durable remission.

Because of issues of toxicity versus efficacy of autologous bone marrow transplantation or peripheral stem cell rescue, the identification of both favorable and unfavorable prognostic markers offers the potential to stratify treatment approaches to DLCL based on risk groups (4-7, 22, 41). The probability of treatment failure remains as high as 25-40 per cent for the most favorable subsets of DLCL based on current prognostic models, highlighting the need

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for genetic or other prognostic markers (5). In addition to its potential diagnostic and prognostic applications, the further identification of BCL-6 breakpoint regions offers the opportunity to develop new polymerase chain reaction-derived measures of minimal residual disease (43). The availability of BCL-6 rearrangement as a new molecular marker of large cell lymphoma constitutes a potentially important clinical tool in the management of patients with this disease.

REFERENCES FOR SECTION V:

1. Simon, R., et al. (1988) The non-Hodgkin's lymphoma pathologic classification project; Longterm follow-up of 1153 patients with non-Hodgkin's lymphoma, Ann. Internal. Med. 109:939-945.
2. Devesa, S.S., and Fears, T. (1992) Non-Hodgkin's lymphoma time trends: United States and international data, Cancer Res. 52:5432-40.
3. Fisher, R.I., et al. (1993) Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma, N. Engl. J. Med. 328:1002-6.
4. Armitage, J.O. (1993) Treatment of non-Hodgkin's lymphoma, N. Engl. J. Med. 328:1023-30.
5. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's lymphoma prognostic factors project, N. Engl. J. Med. 329:987-94.
6. Gulati, S.C., et al. (1988) Autologous bone marrow transplantation for patients with poor-prognosis lymphoma, J. Clin. Oncol. 6:1303-13.

-91-

7. McMaster, M.L., et al. (1991) Results of treatment with high intensity, brief duration chemotherapy in poor prognosis non-Hodgkin's lymphoma, Cancer 68:233-41.
8. Offit, K., and Chaganti, R.S.K. (1991) Chromosomal aberrations in non-Hodgkin's lymphoma: biological and clinical correlations, Hematol. Oncol. Clin. North Am. 5:853-869.
9. McKeithan, T.W. (1990) Molecular biology of non-Hodgkin's lymphomas, Semin. Oncol. 1:30-42.
9. Yunis, J.J., (1989) bcl-2 and other genomic alterations in the prognosis of large-cell lymphoma, N. Engl. J. Med. 320:1047-54.
10. Offit, K., et al. (1989) 18q21 rearrangement in diffuse large cell lymphoma: incidence and clinical significance, Br. J. Haematol. 72:178-83.
11. Jacobson, J.O., et al. (1993) bcl-2 rearrangements in de novo diffuse large cell lymphoma, Cancer 72:231-6.
12. Offit, K., et al. (1991) Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations, Blood 77:1508-15.
13. Offit, K., et al. (1989) t(3;22)(q27;q11): A novel translocation associated with diffuse non-Hodgkin's lymphoma, Blood 74:1876-79.
14. Bastard, C., et al. (1992) Translocations involving band 3q27 and Ig gene regions in non-Hodgkin's lymphoma, Blood 79:2527-31.
15. Ye, B.H., et al. (1993) Cloning of bcl6, the locus involved in chromosome translocations affecting band

-92-

3q27 in B-cell lymphoma, Cancer Res. 53:2732-35.

16. Baron, B.W., et al. (1993) Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas, Proc. Natl. Acad. Sci. 90:5262-66.
17. Kerckaert, J.-P., et al. (1993) LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas, Nat. Genet. 5:66-70.
18. Ye, B.H., et al. (1993) Alterations of a novel zinc-finger encoding gene, bcl-6, in diffuse large-cell lymphoma, Science 262:747-750.
19. The non-Hodkin's lymphoma pathologic classification project. National Cancer Institute sponsored study of classification of non-Hodkin's lymphomas: summary and description of a working formulation for clinical usage, Cancer 49:2112-35.
20. Offit, K., et al. (1991) Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodkin's lymphoma: correlations between recurrent aberrations, histology, and exposure to cytotoxic treatment, Genes Chromosom. Cancer 3:189-201.
21. Danieau, L., et al. (1986) Predictive model for prognosis in advanced diffuse histiocytic lymphoma, Cancer Res. 46:5372-79.
22. Carbone, P.P., et al. (1971) Report of the committee on Hodgkin's disease staging classification, Cancer Res. 31:1860-61.
23. Kempin, S., et al. (1983) Combined modality therapy of

-93-

advanced nodular lymphomas, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 2:56.

- 5 24. Carrato, A., et al. (1987) Randomized comparison of CHOP versus Bleo CHOP for the treatment of diffuse NHL, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 6:779.
- 10 25. Lowenthal, D.A., et al. (1987) The NHL-7 protocol: Alternating non-cross resistant chemotherapy containing methyl-GAG for diffuse non-Hodkin's lymphoma, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 6:778.
- 15 26. Warrell, R.P., et al. (1989) Short term intensive treatment of intermediate-grade non-Hodkin's lymphoma using infusional chemotherapy, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 8:1054.
- 20 27. Straus, D.H., et al. (1991) Small non-Cleaved cell lymphoma (undifferentiated lymphoma/Burkitt's type) in American adults. Results with treatment designed for acute lymphoblastic leukemia in adults, Am. J. Med. 90:328-337.
- 25 28. O'Brien, J., et al. (1992) NHL-15 protocol for diffuse aggressive lymphomas: a dose intense regimen of doxorubicin, vincristine, and cyclophosphamide, Blood 80:157a.
- 30 29. Kaplan, E.L., and Meier, P. (1958) Nonparametric estimation from incomplete observations, J. Am. Stat. Soc. 53:457-81.
- 35 30. Cox, D.R. Regression models and life tables, J. R. Stat. Soc. B. 34:187-220.
31. Mitelman, F. (1991) Catalog of chromosome aberrations in cancer, New York.

-94-

32. Leroux, D., et al. (1990) Translocation t(3;22)(q27;q11) in three patients with diffuse large B cell lymphoma, Leukemia 4:373-376.
- 5 33. Fichelson, S., et al. (1992) Evi-1 expression in leukemic patients with rearrangements of the 3q25-q28 chromosomal region, Leukemia 6:93-99.
- 10 34. Ladanyi M, et al. (1992) Follicular lymphoma with t(8;14)(q24;q32). A distinct clinical and molecular subset of t(8;14)-bearing lymphomas, Blood 79:2124-30.
- 15 35. Raghoebier, S., et al. (1991) Essential differences in oncogene involvement between primary nodal and extranodal large cell lymphoma, Blood 78:2680-86.
- 20 36. Clark, H.M., et al. (1992) Cytogenetic and molecular studies of t(8;14) and t(14;19) in nodal and extranodal b:cell lymphoma, J. Pathol. 166:129-37.
- 25 37. Van Krieken, J.H.J.M., et al. (1991) Molecular genetics of gastrointestinal non-Hodkin's lymphomas: unusual prevalence and pattern of c-myc rearrangements in aggressive lymphomas, Blood 76:797-800.
- 30 38. Romaguera, J.E., et al. (1993) The clinical relevance of t(14;18)/bcl-2 rearrangement and del 6q in diffuse large cell lymphoma and immunoblastic lymphoma, Ann. Oncol. 4:51-4.
- 35 39. Rudders, R.A., et al. (1978) Primary extranodal lymphoma: Response to treatment and factors influencing prognosis. Cancer 42:406-16.
40. Coiffier, B., et al. (1991) Prognostic factors in aggressive malignant lymphomas: description and validation of a prognostic index that could identify patients requiring a more intensive therapy, J. Clin.

-95-

Oncol. 9:211-19.

- 5 41. Schneider, A.M., et al. (1990) Treatment results with an aggressive chemotherapeutic regimen (MACOP-B) for intermediate and some high grade non-Hodkin's lymphomas, J. Clin. Oncol. 8:94-102.
- 10 42. Gribben, J.G., et al. (1993) Detection by polymerase chain reaction of residual cells with the BCl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for b-cell lymphoma, Blood 81:3449-87.

Experimental Detail Section VI

Introduction

5 The *bcl-6* proto-oncogene encodes a POZ/Zinc finger transcriptional repressor expressed in germinal center (GC) B and T cells and required for GC formation and antibody affinity maturation. Deregulation of *bcl-6* expression by chromosomal rearrangements and point mutations of the *bcl-6* promoter region are implicated in the pathogenesis of B-cell lymphoma. The signals regulating *bcl-6* expression are not known. Here the antigen receptor activation leads to BCL-6 phosphorylation by mitogen-activated protein kinase (MAPK) is shown. Phosphorylation, in turn, targets BCL-6 for rapid degradation by the ubiquitin/proteasome pathway. These findings indicate that BCL-6 expression is directly controlled by the antigen receptor via MAPK activation. This signaling pathway may be crucial for the control of B-cell differentiation and antibody response and has implications for the regulation of other POZ/Zinc finger transcription factors in other tissues.

The *bcl-6* proto-oncogene was identified by virtue of its involvement in chromosomal translocations in diffuse large cell lymphoma (DLCL), the most common form of non-Hodgkin's lymphoma (NHL) (Baron et al., 1993; Kerckaert et al., 1993; Ye et al., 1993; Miki et al., 1994). Subsequent studies have demonstrated that rearrangements of the *bcl-6* gene can be found in 30-40% of DLCL and in a minority (5-10%) of follicular lymphoma (FL) (Bastard et al., 1994; LoCoco et al., 1994; Otsuki et al., 1995). These rearrangements juxtapose heterologous promoters, derived from other chromosomes, to the *bcl-6* coding domain, causing its deregulated expression by a mechanism called promoter substitution (Ye et al., 1995; Chen et al., 1998). The 5' noncoding region of the *bcl-6* gene can also be altered by somatic point mutations that are detectable, independent of rearrangements, in ~70% DLCL, 45% FL and AIDS-associated

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NHL (Migliazza et al., 1995; Gaidano et al., 1997). Taken together, rearrangements and mutations of the *bcl-6* promoter region represent the most frequent genetic alteration in human B-cell malignancies, suggesting that they may be important for tumorigenesis (Dalla-Favera et al., 1996).

The BCL-6 protein is a nuclear phosphoprotein belonging to the POZ/Zinc finger (ZF) family of transcription factors (Kerckaert et al., 1993; Ye et al., 1993; Miki et al., 1994). It contains six Krüppel-type carboxy-terminal zinc finger (ZF) motifs that have been shown to recognize specific DNA sequences in vitro (Chang et al., 1996; Seyfert et al., 1996) and an amino-terminal POZ motif (Albagli et al., 1995) shared by various ZF molecules including the *Drosophila* developmental regulators *Tramtrack* and *Broad-complex* (Harrison and Travers, 1990; DiBello et al., 1991), the human KUP (Chardin et al., 1991), ZID (Bardwell and Treisman, 1994) and PLZF (Chen et al., 1993) proteins as well as by POX viruses (Koonin et al., 1992) and the actin-binding *Drosophila* oocyte protein *Kelch* (Xue and Cooley, 1993). BCL-6 functions as a potent transcriptional repressor by binding to its DNA target sequence (Deweindt et al., 1995; Chang et al., 1996; Seyfert et al., 1996).

BCL-6 is an important regulator of lymphoid development and function. In the B-cell lineage, the BCL-6 protein is found only in B cells within germinal centers (GC), but not in pre-B cells or in differentiated progeny such as plasma cells. In the T-cell lineage, BCL-6 protein is detectable in cortical thymocytes and in CD4⁺ T cells within GC as well as scattered in the perifollicular area (Cattoretti et al., 1995; Onizuka et al., 1995; Allman et al., 1996). Mice deficient in BCL-6 display normal B-cell, T-cell and lymphoid organ development, but have a selective defect in T-cell-dependent antibody responses because of the inability of follicular B cells to proliferate and form GC

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(Dent et al., 1997; Ye et al., 1997). In addition, BCL-6-deficient mice develop an inflammatory response in multiple organs characterized by infiltration of eosinophils and IgE-bearing B lymphocytes typical of a Th2-mediated inflammatory response. These phenotypes may be explained by the ability of BCL-6 to bind the STAT-6 DNA-binding site and repress transcription activated by STAT-6, the main nuclear effector of IL-4 signaling (Dent, et al., 1997; Ye et al. 1997).

The expression and requirement of BCL-6 during GC formation and its alteration in GC-derived lymphoma suggest that BCL-6 may be a key regulator of GC development and antibody-mediated immune response. Toward the elucidation of the signals that regulate GC expression, we report here the identification of a signaling pathway by which B-cell antigen receptor directly regulates BCL-6 stability.

The BCL-6 gene encodes a POZ/Zinc finger protein which functions as a sequence-specific transcriptional repressor expressed in B cells and CD4+ T cells within germinal centers (GC) and downregulated in post-GC B cells. Inactivation of BCL-6 in mice demonstrated that it is required for GC formation, antibody affinity maturation and balanced required for GC formation, antibody affinity maturation and balanced required for GC formation, antibody affinity maturation and balanced Th-2-mediated response. BCL-6 gene expression is commonly deregulated in diffuse large cell lymphoma (DLCL) and, less frequently, in follicular lymphoma (FL) by rearrangements and mutations of its 5' non-coding region. The mechanism regulating the specific pattern of expression of BCL-6 in GC lymphocytes is unknown. We show that BCL-6 can be phosphorylated by mitogen activated protein kinase (MAPK) in vitro as well as in vivo in 293T cells transfected with vectors expressing BCL-6 and constitutively activated MAPK kinase (MEK-2E). MAPK kinase expression leads to rapid degradation of BCL-6, which does not occur in the presence of inactive MEK or

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when BCL-6 phosphorylation mutants are used as targets, indicating that degradation is dependent upon phosphorylation. Phosphorylation-dependent degradation is mediated by the ubiquitin/proteasome pathway since it is prevented by MG132, a specific inhibitor, but not by inhibitors of other proteolytic pathways; in addition, ubiquitin-BCL-6 conjugates can be detected by immunoprecipitation. To demonstrate the physiologic significance of MAPK-mediated phosphorylation/degradation of BCL-6 in B cells, a B cell lymphoma cell line (Ramos) was treated with anti-IgM antibodies with mimics B cell antigen receptor signaling and specifically activates (ERK2) MAPK. This treatment led to rapid BCL-6 phosphorylation followed by ubiquitin/proteasome-dependent degradation. These results identify a MAPK-mediated signaling pathway by which antigen receptor activation causes inactivation of the BCL-6 transcription factor. Since BCL-6 is required for GC formation, this pathway may be critical for the antigen-driven post-GC differentiation of B cells into immunoblasts or memory cells.

Materials and Methods

Reagents and Plasmids

Goat anti-human IgM(μ -heavy chain specific) was obtained from Southern Biotechnology. Polyclonal anti-BCL-6 (N-70-6) antiserum was produced by using the amino-terminal peptides of BCL-6 (Cattoretti et al., 1995). Monoclonal mouse anti-ERK2(C-14) was purchased from Santa Cruz Biotechnology. (Santa Cruz, CA). Monoclonal mouse anti-ubiquitin was obtained from Zymed laboratories, Inc.(South San Francisco, CA). Monoclonal mouse anti-HA (12CA5) was purchased from Boehringer Mannheim, as was Calpain Inhibitor II. Protein A-Sepharose CL-4B and glutathione-Sepharose were purchased from Pharmacia. Myelin basic protein (MBP) and N-CBZ-Leu-Leu-Leu-AL (MG132) were obtained from Sigma. PD098059 was purchased from Calbiochem-Novabiochem (La Jolla, CA). The GST-BCL6,

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GST-BCL6ΔZF, GST-BCL6ZF, GST-BCL6ΔZF_{Ala333} and GST-BCL6ΔZF_{Ala333,343} fusion proteins were produced by pGEX-2TK-based plasmids (Pharmacia Biotech) containing full-length, deletion or point mutants of *bcl-6*. The point mutations (Ala333, Ala343) were generated by PCR-based methods; the sequence of the resulting plasmids was confirmed by nucleotide sequence analysis. pMT2T-BCL-6 and B6BS-TK-LUC have been described as previous (Chang et al., 1996). pMT2T-BCL-6_{Ala333,343} was constructed by transferring the *BclI*-*NcoI* fragments of plasmid pGEX-2TK-BCL6ΔZF_{Ala333,343} into the pMT2T-BCL-6 vector. MEK-2E-EE-CMV and MEK-EE-CMV for expressing of constitutively active or inactive MEK were provided by D. Templeton (Case Western Reserve University, Cleveland, OH). The pMT2T-HA-BCL-6, pMT2T-HA-BCL-6Δ(300-417), and pMT2T-HA-BCL-6ZF vectors were constructed by inserting the sequences encoding the HA epitope upstream and in frame with *bcl-6* coding sequences. Deletion mutants of *bcl-6* were produced by PCR-based methods and confirmed by sequencing. His₆-ubiquitin-CMV was kindly provided by T. Maniatis (Harvard Medical School, Boston, MA). Episomally replicating plasmid, pHeBo-MT which carries EBV oriP, hygromycin B and MT promoter efficiently yields hygromycin-resistant colonies.

ERK2 kinase assays

BCL-6 GST fusion proteins were purified using glutathione-Sepharose beads as suggested by the manufacturer (Pharmacia Biotech). Recombinant ERK2 (New England Biolabs) assays were performed as suggested by the manufacturer using purified wild-type and mutant GST fusion proteins as substrates. In solid-phase ERK2 kinase assays, cells were lysed in ice-cold lysis-buffer (50 mM Tris at pH7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM ZnCl₂, 1 mM Na₃O₄, 10 mM EGTA, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml of leupeptin, and 1 μg/ml pepstatin) and centrifuged at 100,000g for 15 min at 4°C. The supernatant (250-500 μg of cellular protein) was then

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immunoprecipitated using anti-ERK2 antibodies (C-14) and Protein A-Sepharose CL-4B. Beads were washed three times with lysis buffer and once with kinase buffer (50 mM Tris, pH7.5, 10 mM MnCl₂, 5 mM MgCl₂). Reactions were initiated by adding 50 μ l of kinase buffer containing substrate MBP, 5 μ M ATP and 5 μ Ci [γ -³²P] ATP. After 15 min at 37C, reactions were terminated by adding 2x SDS-PAGE sample buffer. Samples were electrophoresed on 15% SDS-polyacrylamide gels which were then dried and analyzed by autoradiography.

Cell transfection

293T cells, grow in DMEM, 10% FBS, were transfected transiently with various DNA vectors using standard calcium phosphate precipitation methods. Ramos cells, grown in IMDM, 10% FBS, were transfected stably with the plasmid pHeBo-MT-HA-BCL-6, pHeBo-MT-HA-BCL-6_{Δ333-343} and the deletion mutant construct pHeBo-MT-HA-BCL-6ZF by electroporation followed by selection in hygromycin B (400 μ g/ml). HA-*bcl-6* gene expression under control of the metallothionein (MT) promoter were induced by adding 1 μ M of CdCl₂.

Northern and Western blot analysis

Total RNA were isolated from cells by using Trizol-reagents (GIBCO-BRL) and equal amounts of RNA were separated on 1% formaldehyde-agarose gel. Northern blot analysis was performed by using standard methods with full-length *bcl-6* cDNA as probes and normalized by GAPDH hybridization. Whole-cell lysates were prepared by lysing cells in RIPA buffer with 2 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml of leupeptin, 1 μ g/ml pepstatin, 1 mM NaVO₃, 5 mM NaF, 10 mM β -glycerophosphate. For transient transfectants, protein amounts loaded on gel were normalized by transfection efficiency (β -gal activity). For Ramos cells and their stable transfectants (untreated or treated), equal amounts

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of protein were analyzed by 8% or 10% SDS-PAGE, and subsequently by Western blot analysis using anti-BCL-6 (N-70-6), anti-ERK2 (C-14) or anti-HA (12CA5) antibodies at 1:3000, 1:1000 or 1:500 dilutions. The results were visualized by ECL (Amersham).

In vivo ubiquitination assay

293T cells were transfected transiently with pMT2T-BCL-6, His₆-ubiquitin-CMV and MEK-2E-CMV vectors as indicated. MG132 (50 μ M) was added 8 hrs after transfection. The total amount of transfected DNA was kept constant in all experiments by adding empty vector. Twenty-four hours after transfection, cells were lysed in RIPA buffer with 10 mM N-ethylmaleimide and various protease inhibitors as described (Pagano et al., 1995). The cell lysates were then immunoprecipitated using anti-BCL-6 antibodies. The immunoprecipitates were loaded on 6% SDS-PAGE and processed for Western blot analysis using the anti-ubiquitin antibodies (Zymed) at 1:1000 dilution as described (Avantaggiati et al., 1996).

Pulse-chase labeling experiment

Ramos cells (12×10^6) were collected by centrifugation, washed in PBS, resuspended in 100ml of DMEM without methionine and cysteine (GIBCO-BRL), and starved for 60 min. [³⁵S]methionine and [³⁵S]cysteine (3mCi; ICN) were added and pulse-labeled for 60 min, and then treated with anti-IgM for 30 min. Cold methionine and cysteine were then added to final concentrations of 150 μ g/ml. Cells were collected and lysed in RIPA buffer with proteinase and phosphatase inhibitors. The cell extracts, adjusted for equal cpm, were immunoprecipitated with anti-BCL-6 antibodies, and analyzed by SDS-PAGE followed by autoradiography.

Experimental Results

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Recent studies have shown that the BCL-6 protein is phosphorylated at multiple sites by mitogen-activated protein kinases (MAPKs), ERK-1 and ERK-2, but not by Jun amino-terminal kinase (JNK) in vitro and in vivo (Moriyama et al., 1997). The results shown in Figure 19 confirm that purified recombinant MAPK (ERK-2) can phosphorylate GST-BCL-6 fusion proteins in vitro. The phosphorylation targets were mapped to the amino-terminal half of the molecule since a carboxy-terminal deletion mutant (GST-BCL-6 Δ ZF) could be phosphorylated at levels comparable to the wild-type molecule, whereas an amino-terminal deletion mutant (GST-BCL-6ZF) could not be phosphorylated at all (Fig. 19B). Because BCL-6 contains two perfect consensus sites (PXSP) for MAPK-mediated phosphorylation (see Fig. 19A), we generated two mutants (BCL-6_{Ala333} and BCL-6_{Ala333,343}) in which one or both of these sites were altered by substituting serines with alanines. These two mutants were phosphorylated at much lower levels than wild type BCL-6, with BCL-6_{Ala333,343} displaying the lowest levels (Fig. 19C). This result indicates that the Ser₃₃₃ and Ser₃₄₃ residues represent a significant fraction, although not all, of the BCL-6 phosphorylation target sites. The residual low level of phosphorylation is consistent with the existence of additional potential MAPK target sequences (SP) clustered within the central domain of the BCL-6 molecule.

MAPK-mediated phosphorylation induces BCL-6 degradation

To determine the effects of MAPK-mediated phosphorylation on *bcl-6* expression and function, 293T cells (which do not express endogenous BCL-6) were cotransfected with vectors expressing BCL-6, and a MEK (MAP/ERK kinase) mutant (MEK-2E) that functions as a constitutively active MAPK kinase (Yan and Templeton, 1994). Western blot analysis of transfected cell extracts showed that MEK-2E expression (documented by increased ERK2 kinase activity of MEK-2E

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transfected cell extracts in solid-phase kinase assays in vitro; Fig. 20A, bottom) induced a dramatic reduction of BCL-6, but not ERK, levels (Fig. 20A, top and middle). The observed reduction in BCL-6 protein levels was dependent upon the phosphorylation activity of MEK-2E, since it did not occur when a vector expressing inactive MEK was cotransfected with *bcl-6* (Fig. 20B). Northern blot analysis of the same transfected cells showed that the reduction in BCL-6 protein levels were not caused by decreased *bcl-6* mRNA levels (Fig. 20B, bottom). Furthermore, the MEK-2E-induced decrease in BCL-6 levels was dependent on target phosphorylation, as the partial phosphorylation-resistant mutant BCL-6_{Ala333,343} was partially resistant to MEK-2E-mediated down-regulation (Fig. 20C). These results indicate that the MEK-2E-induced decrease in BCL-6 levels is not caused by decreased gene transcription or protein synthesis, but rather by decreased protein stability.

Consistent with the MEK-2E-induced reduction in BCL-6 levels, a transient cotransfection assay in 293T cells showed that MEK-2E, but not MEK, could eliminate the transcriptional transrepressor activity of wild-type BCL-6 (Fig. 20D, lanes 3-5) on a reporter vector expressing the luciferase gene downstream to the BCL-6 DNA-binding site (B6BS) (Chang et al., 1996); the partial phosphorylation-resistant mutant BCL-6_{Ala333,343} was partially resistant to MEK-2E (Fig. 20D, lanes 12-14). Overall, these results indicate that MAPK activation leads to functional inactivation of BCL-6 by causing its accelerated degradation.

BCL-6 degradation is mediated by ubiquitin/proteasome pathway

In examining the possible mechanisms for MAPK-mediated degradation of BCL-6, we noted that the cluster of MAPK putative phosphorylation sites are embedded in a region

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enriched in proline, glutamine and serine, within which we identified three typical PEST sequences that score 9.4, 5.0, and 2.6, respectively (Fig. 21A; any score above zero denotes a possible PEST region; scores greater than five indicate the strongest candidates). These motifs have been demonstrated to represent targets for regulated protein degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). To determine whether MAPK-mediated BCL-6 degradation targeted these PEST sequences, constructed vectors expressing two epitope HA-tagged *bcl-6* deletion mutants (see Fig. 21A) and co-transfected them with the MEK-2E vector into 293T cells. Western blot analysis using anti-HA antibodies (Fig. 21B) showed that MEK-2E-mediated degradation targeted the amino-terminal half of the molecule and it was completely abolished in the BCL-6Δ(300-417) internal deletion mutant that lacks a small portion of the BCL-6 protein containing all three PEST sequences. These results indicate that MAPK-induced phosphorylation and degradation of BCL-6 target PEST sequences located in the same domain as the MAPK phosphorylation sites.

The involvement of PEST sequences suggested that MAPK-induced BCL-6 degradation could be mediated by the ubiquitin/proteasome pathway (Hochstrasser, 1996). Therefore, we tested whether MEK-2E mediated degradation of BCL-6 in transfected 293T cells could be inhibited by the proteasome inhibitor Cbz-LLL (MG132) (Kim and Maniatis, 1996; Palombella et al., 1994; Rock et al., 1994). Figure 22A shows that BCL-6 degradation was completely inhibited by MG132, but not by DMSO (solvent control) or calpain inhibitor II (CI II), a cysteine-protease inhibitor (Kim and Maniatis, 1996; Palombella et al., 1994). Because the addition of multiple ubiquitins to the proteolysis substrate is a key step preceding target degradation by the proteasome, we then tested whether BCL-6/ubiquitin conjugates in vivo could be detected. To this end, 293T cells were transfected with vectors expressing BCL-6,

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MEK-2E and epitope (His₆)-tagged ubiquitin in the presence or absence of MG132. Cell lysates were subjected to immunoprecipitation with anti-BCL-6 antibodies, and the immunoprecipitates were analyzed by Western blotting using anti-ubiquitin antibodies. Fig. 22B shows that in the absence of MG132, low levels of BCL-6/ubiquitin were detectable when BCL-6 and ubiquitin were co-expressed with exogenous MEK-2E (lane 4); in the presence of MG132, typical ladders representing multi-ubiquitinated forms of BCL-6 were detectable at high levels in the presence of MEK-2E (lane 8); low levels were detectable also in its absence (lane 7), suggesting that the normal turn-over of BCL-6 degradation may be mediated by basal levels of endogenous MAPK activity. Based on the specific pharmacological inhibition and the detection of MEK-2E-inducible BCL-6/ubiquitin conjugates, we conclude that MAPK-induced phosphorylation induces degradation of BCL-6 via the ubiquitin/proteasome pathway.

MAPK-mediated phosphorylation and degradation of BCL-6 is induced by antigen-receptor signaling in B Cells

To demonstrate the physiological significance of MAPK-mediated phosphorylation/degradation of BCL-6 in B cells, we treated a B-cell lymphoma cell line (Ramos) with anti-IgM antibodies, a treatment that mimics B-cell antigen-receptor signaling and specifically activates MAPK (ERK2) (Gold et al., 1992; Sakata et al., 1995; Sutherland et al., 1996). As previously demonstrated, an in vitro assay showed that ERK2 kinase activity was rapidly increased 5 min after anti-IgM treatment (Fig. 23A); this was followed by hyperphosphorylation of ERK2 and BCL-6 (note the slow migrating bands in Fig. 23A) and by the disappearance of BCL-6, but not ERK2. In the same experiment, Northern blot analysis showed that *bcl-6* mRNA levels did not change during anti-IgM treatment of Ramos cells (Fig. 23A, bottom). In order to determine whether hyperphosphorylation was associated with increased BCL-6

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instability, we analyzed the half-life of BCL-6 in anti-IgM-treated Ramos cells by a "pulse-chase" labeling experiment. The results (Fig. 23B) showed that the hyperphosphorylated (slow-migrating) forms of BCL-6 were significantly less stable than the hypophosphorylated (fast-migrating) forms (half life 4-6 hrs.). Anti-IgM-induced BCL-6 degradation was dependent upon phosphorylation since it was inhibited by a specific MAPK inhibitor PD098059 (Fig. 23C) (Dudley et al., 1995; Pang et al., 1995), and was mediated by the ubiquitin/proteasome pathway since was specifically inhibited by MG132 (Fig. 23D). Finally, anti-IgM treatment of Ramos cells stably transfected with Cadmium-inducible vectors expressing HA-tagged wild-type, 333/343 mutant, or amino-terminal deleted BCL-6 proteins showed that degradation required phosphorylation of the 333 and 343 serines as well as the amino-terminal half of BCL-6 containing the PEST motifs (Fig. 23E). These results demonstrate that MAPK-mediated phosphorylation of BCL-6 and its degradation by the ubiquitin/proteasome pathway represent a physiologic pathway that can be activated by antigen receptor signaling in B cells.

Experimental Discussion

The present study identifies a signal transduction pathway by which the antigen receptor regulates the stability of the BCL-6 transcription factor in B cells. The results have implications for the normal mechanism regulating GC formation as well as for the role of deregulated *bcl-6* expression in lymphomas deriving from GC B cells. In addition, several observations suggest that MAPK-mediated regulation of POZ/Zinc finger protein stability may represent a general, highly conserved regulatory mechanism in eukaryotic cells.

Regulation of BCL-6 stability during GC formation

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The finding that antigen-receptor-induced activation of MAPK leads to BCL-6 degradation must be seen in the context of the complex network of signals modulating receptor signaling in GC B cells (Tedder, et al., 1997; Cambier, 1997). During GC formation, activation of this pathway is consistent with the observation that pre-GC B cells in the follicular mantle zone, the site where B cells encounter the antigen, express *bcl-6* RNA, but not the BCL-6 protein (Allman et al., 1996; Cattoretti et al., 1995). Within the GC, the coexistence of antigen and *bcl-6* expression implies that antigen-receptor signaling must be modulated by mechanisms that allow BCL-6 stability. These mechanisms may include down-regulation of antigen-receptor expression in centroblasts (MacLennan, 1994), modulation of receptor signaling by CD22 or Fc γ receptor (Tedder, et al., 1997; Cambier, 1997), and the activity of de-ubiquitinases (DUB), which regulate substrate ubiquitination and are induced by cytokines acting on GC B cells (Zhu et al., 1996). During post-GC differentiation, antigen-induced degradation may serve as a rapid mechanism to down-regulate *bcl-6* expression, in synergy with transcriptional down-regulation by CD40 signaling (Allman et al., 1996; Cattoretti et al., 1997). Finally, the regulation of BCL-6 stability during GC development is likely to be affected by various additional signals that activate MAPK in B cells including various cytokines (TNF, IL-6, IL-2) (Vieter et al., 1993; Minami et al., 1994; Fukada et al., 1996). The effect of these signals on the pathway linking the antigen receptor to BCL-6 can be tested in the experimental systems used in this study.

Implication for lymphomagenesis

Most B-cell lymphoma types, including follicular (FL), diffuse large cell, (DLCL) and Burkitt (BL) lymphoma, are thought to derive from the GC B cells. Although rearrangements and/or mutations of the *bcl-6* regulatory region are found most frequently in DLCL and FL, all

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GC-derived lymphoma, including those carrying a structurally normal *bcl-6* gene, express the BCL-6 protein (Cattoretti et al., 1995). This implies that the BCL-6 protein is stable in tumor cells and suggests that
5 MAPK-mediated degradation may be blocked by genetic or epigenetic alterations affecting the pathway leading to BCL-6 degradation. The observation that BCL-6 degradation can be triggered from the cell surface by activation of the antigen receptor has potential relevance for the therapy of
10 B-cell lymphoma.

MAPK-mediated regulation of POZ/Zinc finger transcription factors

15 MAPK is a ubiquitous, evolutionarily conserved signal transducer that is activated by heterogeneous signals that originate from the cell membrane and are transduced to MAPK via RAS proteins (Gold and Matsuuchi, 1995; Alberola-Ila et al., 1997). Accordingly, POZ/zinc finger proteins
20 represent a large family of highly conserved transcription factors including *Drosophila* cell fate regulators such as *Tramtrack* and *Broad-complex*, as well as human cancer-associated proteins such as BCL-6 and PLZF. These molecules have strong structural (POZ and ZF domains), as
25 well as functional homologies being transcriptional repressors that control cell differentiation (Albagli et al., 1995; Chen et al., 1994; Emery et al., 1994). Most notably, POZ/zinc finger proteins also carry possible MAPK phosphorylation sites and PEST sequences in approximately
30 the same position as those carried by BCL-6 (Niu et al., unpublished observation). In *Drosophila*, degradation of TTK88, a POZ/zinc finger inhibitor of neural-cell differentiation, has been shown to be mediated by MAPK (Li et al., 1997; Tang et al., 1997). Thus, degradation of
35 POZ/zinc finger transcription factors may represent a general mechanism by which the RAS/MAPK pathway controls cell function and differentiation.

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References for Experimental Detail Section VI

- 5 Albagli, O., Dhordain, P., Deweindt, C., Lecocq, G., and
Leprince, D. 1995. The BTB/POZ domain: a new
protein-protein interaction motif common to DNA- and
actin-binding proteins. *Cell Growth & Differ.* 6: 1193-1198.
- 10 Alberola-Ila, J., Takaki, S., Kerner, J. D., and
Perlmutter, R. M. 1997. Differential signaling by
lymphocyte antigen receptors. *Annu. Rev. Immunol.* 15:
125-154.
- 15 Allman, D., Jain, A., Dent, A., Maile, R. R., Selvaggi, T.,
Kehry, M. R., and Staudt, L. M. 1996. BCL-6 expression
during B-cell activation. *Blood* 87: 5257-5268.
- 20 Avantaggiati, M., Carbone, M., Graessman, A., Nakatani, Y.,
Howard, B., and Levine, A. 1996. The SV40 large T antigen
and adenovirus E1A oncoproteins interact with distinct
isoforms of the transcriptional co-activator p300. *EMBO J.*
15: 2236-2248.
- 25 Bardwell, V. J., and Treisman, R. 1994. The POZ domain: a
conserved protein-protein interaction motif. *Genes & Dev.*
8: 1664-1677.
- 30 Baron, B. W., Nucifora, G., McCabe, N., Espinosa, R. d.,
Le, B. M., and McKeithan, T. W. 1993. Identification of the
gene associated with the recurring chromosomal
translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in
B-cell lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* 90:
5262-5266.
- 35 Bastard, C., Deweindt, C., Kerckaert, J. P., Lenormand, B.,
Rossi, A., Pezzella, F., Fruchart, C., Duval, C.,
Monconduit, M., and Tilly, H. 1994. LAZ3 rearrangements in
non-Hodgkin's lymphoma: correlation with histology,
immunophenotype, karyotype, and clinical outcome in 217

-111-

patients. *Blood* 83: 2423-2427.

5 Cambier, J. C. 1997. Positive and negative signal co-operativity in the immune system: the BCR, Fc gamma RIIB, CR2 paradigm. *Biochemical Society Transactions* 25: 441-445.

10 Cattorette, G., Chang, C. C., Cechova, K., Zhang, J., Ye, B. H., Falini, B., Louie, D. C., Offit, K., Chaganti, R. S., and Dalla-Favera, R. 1995. BCL-6 protein is expressed in germinal-center B cells. *Blood* 86: 45-53.

15 Cattorette, G., Zhang, J., Cleary, A. M., Lederman, S., Gaidano, G., Carbone, A., Chaganti, R. S. K., and Dalla-Favera, R. 1997. Downregulation of BCL-6 gene expression by CD40 and EBV latent membrane protein-1 (LMP1) and its block in lymphoma carrying BCL-6 rearrangements. *Blood* 90: Supplement 1, p175a.

20 Chang, C. C., Ye, B. H., Chaganti, R. S., and Dalla-Favera, R. 1996. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc. Natl. Acad. Sci. U.S.A.* 93: 6947-6952.

25 Chardin, P., Courtois, G., Mattei, M. G., and Gisselbrecht, S. 1991. The KUP gene, located on human chromosome 14, encodes a protein with two distant zinc fingers. *Nucleic Acid Res.* 19: 1431-1436.

30 Chen, W., Iida, S., Louie, D. C., Dalla-Favera, R., and Chaganti, R. S. K. 1998. Heterologous Promoters Fused to BCL-6 by Chromosomal translocations affecting band 3q27 cause its deregulated expression during B-cell differentiation. *Blood* 91: 603-607.

35 Chen, Z., Brand, N. J., Chen, A., Chen, S. J., Tong, J. H., Wang, Z. Y., Waxman, S., and Zelent, A. 1993. Fusion between a novel Kruppel-like zinc finger gene and the

-112-

retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. *EMBO J.* 12: 1161-1167.

- 5 Chen, Z., Guidez, F., Rousselot, P., Agadir, A., Chen, S. J., Wang, Z. Y., Degos, L., Zelent, A., Waxman, S., and Chomienne, C. 1994. PLZF-RAR alpha fusion proteins generated from the variant t(11;17)(q23;q21) translocation in acute promyelocytic leukemia inhibit ligand-dependent
10 transactivation of wild-type retinoic acid receptors. *Proc. Natl. Acad. Sci. U.S.A.* 91: 1178-1182.

- Dalla-Favera, R., Ye, B. H., Cattoretti, G., Lo Coco, F., Chang, C. C., Zhang, J., Migliazza, A., Cechova, K., Niu, H., Chaganti, S., Chen, W., Louie, D. C., Offit, K., and
15 Chaganti, R. S. 1996. BCL-6 in diffuse large-cell lymphomas. In: *Important Advances in Oncology* (V.T. DeVita, S. Hellman, and S.A. Rosenberg, eds.), pp. 139-148. Lippincott-Raven Publishers, Philadelphia.

- 20 Dent, A. L., Shaffer, A. L., Yu, X., Allman, D., and Staudt, L. M. 1997. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* 276: 589-592.

- 25 Deweindt, C., Albagli, O., Bernardin, F., Dhordain, P., Quief, S., Lantoine, D., Kerckaert, J. P., and Leprince, D. 1995. The LAZ3/BCL6 oncogene encodes a sequence-specific transcriptional inhibitor: a novel function for the BTB/POZ
30 domain as an autonomous repressing domain. *Cell Growth & Differ.* 6: 1495-503.

- DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W., and Guild, G. M. 1991. The *Drosophila*
35 *Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* 129: 385-397.

Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and

-113-

Saltiel, A. R. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.* 92: 7686-7689.

5 Emery, I. F., Bedian, V., and Guild, G. M. 1994. Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* 120: 3275-3287.

10

Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K., and Hirano, T. 1996. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3
15 in anti-apoptosis. *Immunity* 5: 449-460.

Gaidano, G., Carbone, A., Pastore, C., Capello, D., Migliazza, A., Gloghini, A., Roncella, S., Ferrarini, M., Saglio, G., and Dalla-Favera, R. 1997. Frequent mutation of
20 the 5' noncoding region of the BCL-6 gene in acquired immunodeficiency syndrome-related non-Hodgkin's lymphomas. *Blood* 89: 3755-3762.

Gold, M. R., and Matsuuchi, L. 1995. Signal transduction by
25 the antigen receptors of B and T lymphocytes. *International Review of Cytology* 157: 181-276.

Gold, M. R., Sanghera, J. S., Stewart, J., and Pelech, S. L. 1992. Selective activation of p42 mitogen-activated
30 protein (MAP) kinase in murine B lymphoma cell lines by membrane immunoglobulin cross-linking. Evidence for protein kinase C-independent and -dependent mechanisms of activation. *Biochem. J.* 287: 269-276.

35 Harrison, S. D., and Travers, A. A. 1990. The *Tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* 9: 207-216.

-114-

Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* 30: 405-439.

- 5 Kerckaert, J. P., Deweindt, C., Tilly, H., Quief, S., Lecocq, G., and Bastard, C. 1993. LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas. *Nature Genetics* 5: 66-70.
- 10 Kim, T. K., and Maniatis, T. 1996. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. *Science* 273: 1717-9.
- 15 Koonin, E. V., Senkevich, T. G., and Chernos, V. I. 1992. A family of DNA virus genes that consists of fused portions of unrelated cellular genes. *Trends Biochem. Sci.* 17: 213-214.
- 20 Li, S., Li, Y., Carthew, R. W., and Lai, Z.-C. 1997. Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor. *Tramtrack Cell* 90: 469-478.
- 25 LoCoco, C. F., Ye, B. H., Lista, F., Corradini, P., Offit, K., Knowles, D. M., Chaganti, R. S., and Dalla, F. R. 1994. Rearrangements of the BCL6 gene in diffuse large cell non-Hodgkin's lymphoma. *Blood* 83: 1757-1759.
- 30 MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117-139.
- 35 Migliazza, A., Martinotti, S., Chen, W., Fusco, C., Ye, B. H., Knowles, D. M., Offit, K., Chaganti, R. S., and Dalla, F. R. 1995. Frequent somatic hypermutation of the 5' noncoding region of the BCL6 gene in B-cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 92: 12520-12524.

-115-

Miki, T., Kawamata, N., Hirose, S., and Aoki, N. 1994. Gene involved in the 3q27 translocation associated with B-cell lymphoma, BCL5, encodes a Kruppel-like zinc-finger protein. *Blood* 83: 26-32.

5

Minami, Y., Oishi, I., Liu, Z. J., Nakagawa, S., Miyazaki, T., and Taniguchi, T. 1994. Signal transduction mediated by the reconstituted IL-2 receptor. Evidence for a cell type-specific function of IL-2 receptor beta-chain. *J. Immunol.* 152: 5680-5690.

10

Moriyama, M., Yamochi, T., Semba, K., Akiyama, T., and Mori, S. 1997. BCL-6 is phosphorylated at multiple sites in its serine- and proline-clustered region by mitogen-activated protein kinase (MAPK) in vivo. *Oncogene* 14: 2465-2474.

15

Onizuka, T., Moriyama, M., Yamochi, T., Kuroda, T., Kazama, A., Kanazawa, N., Sato, K., Kato, T., Ota, H., and Mori, S. 1995. BCL-6 gene product, a 92- to 98-kD nuclear phosphoprotein, is highly expressed in germinal center B cells and their neoplastic counterparts. *Blood* 86: 28-37.

20

Otsuki, T., Yano, T., Clark, H. M., Bastard, C., Kerckaert, J. P., Jaffe, E. S., and Raffeld, M. 1995. Analysis of LAZ3 (BCL-6) status in B-cell non-Hodgkin's lymphomas: results of rearrangement and gene expression studies and a mutational analysis of coding region sequences. *Blood* 85: 2877-2884.

25

Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269: 682-685.

30

35

Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. 1994. The ubiquitin-proteasome pathway is

-116-

required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell* 78: 773-785.

5 Pang, L., Sawada, T., Decker, S. J., and Saltiel, A. R. 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.* 270: 13585-13588.

10 Rechsteiner, M., and Rogers, S. W. 1996. PEST sequences and regulation by proteolysis. *Trends in Biochem. Sci.* 21: 267-271.

15 Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78: 761-771.

20 Rogers, S., Wells, R., and Rechsteiner, M. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234: 364-368.

25 Sakata, N., Patel, H. R., Terada, N., Aruffo, A., Johnson, G. L., and Gelfand, E. W. 1995. Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. *J. Biol. Chem.* 270: 30823-30828.

30 Seyfert, V. L., Allman, D., He, Y., and Staudt, L. M. 1996. Transcriptional repression by the proto-oncogene BCL-6. *Oncogene* 12: 2331-2342.

35 Sutherland, C. L., Heath, A. W., Pelech, S. L., Young, P. R., and Gold, M. R. 1996. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *J. Immunol.* 157: 3381-3390.

Tang, A. H., Neufeld, T. P., Kwan, E., and Rubin, M. 1997.

-117-

PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism. *Cell* 90: 459-467.

- 5 Tedder, T. F., Tuscano, J., Sato, S., and Kehrl, J. H. 1997. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. *Annu. Rev. Immunol.* 15: 481-504.
- 10 Vietor, I., Schwenger, P., Li, W., Schlessinger, J., and Vilcek, J. 1993. Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. *J. Biol. Chem.* 268: 18994-9.
- 15 Xue, F., and Cooley, L. 1993. Kelch encodes a component of intercellular bridges in *Drosophila* egg chamber. *Cell* 72: 681-693.
- 20 Yan, M., and Templeton, D. J. 1994. Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. *J. Biol. Chem.* 269: 19067-19073.
- 25 Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S. K., Rothman, P., Stall, A. M., Pandolfi, P. P., and Dalla-Favera, R. 1997. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type
30 inflammation. *Nature Genetics* 16: 161-170.
- 35 Ye, B. H., Chaganti, S., Chang, C. C., Niu, H., Corradini, P., Chaganti, R. S., and Dalla, F. R. 1995. Chromosomal translocations cause deregulated BCL6 expression by promoter substitution in B cell lymphoma. *EMBO J.* 14: 6209-6217.
- Ye, B. H., Lista, F., Lo Coco, F., Knowles, D. M., Offit,

-118-

K., Chaganti, R. S., and Dalla-Favera, R. 1993. Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. *Science* 262: 747-750.

- 5 Zhu, Y., Carroll, M., Papa, F. R., Hochstrasser, M., and D'Andrea, A. D. 1996. DUB-1, a deubiquitinating enzyme with growth-suppressing activity. *Proc. Natl. Acad. Sci. U.S.A.* 93: 3275-3279.

10

-119-

What is claimed is:

1. A method of degrading BCL-6 in cells comprising:
5 administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation.
2. The method of claim 1 wherein the molecule which
10 induces phosphorylation of the BCL-6 is a mitogen-activated protein kinase (MAPK).
3. The method of claim 1 wherein the molecule which
15 induces phosphorylation of the BCL-6 is a functionally active mutant of a mitogen-activated protein kinase (MAPK).
4. The method of claim 2 wherein the MAPK is ERK-1 or ERK-2.
- 20 5. The method of claim 1, wherein the BCL-6 is phosphorylated either at one site or at multiple sites.
- 25 6. The method of claim 1, wherein the molecule which induces phosphorylation of the BCL-6 is a molecule which activates an antigen receptor on B cell surfaces.
- 30 7. The method of claim 6, wherein the molecule which activates an antigen receptor on B cell surfaces is an antibody.
8. The method of claim 7, wherein the antibody is an anti-IgM antibody.
- 35 9. The method of claim 6, wherein the molecule which activates an antigen receptor on B cell surfaces is a molecule which activates MAPK in B cells.

-120-

10. The method of claim 9, wherein the molecule which activates MAPK in B cells is a cytokine.

11. The method of claim 10, wherein the cytokine is TNF, IL-6, or IL-2.

12. The method of claim 1, wherein the molecule is cross-linked to a B cell antigen receptor to activate the receptor.

13. The method of claim 1, wherein cross-linking the molecule to the B cell antigen receptor activates the MAPK.

14. A method of treating a subject with lymphoma which comprises:

administering an effective amount of a pharmaceutical composition comprising a molecule which induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable carrier, thereby treating the subject with lymphoma.

15. The method of claim 14, wherein the lymphoma expresses BCL-6.

16. The method of claim 14, wherein the pharmaceutical composition comprises a MAPK activator.

17. The method of claim 16, wherein the MAPK activator is an antibody.

18. The method of claim 17, wherein the antibody is an anti-IgM antibody.

19. The method of claim 16, wherein the MAPK activator is a cytokine.

-121-

20. The method of claim 19, wherein the cytokine is TNF, IL-6, or IL-2.
21. The method of claim 14, wherein the lymphoma is a B-cell lymphoma.
22. The method of claim 21, wherein the B-cell lymphoma is derived from germinal center B cells.
23. The method of claim 14, wherein the administration of the pharmaceutical composition is intravenous or intratumor.
24. A method of regulating decreasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents expression of BCL-6 protein so as to thereby decreasing BCL-6 levels in the cells.
25. The method of claim 24, wherein the compound which interferes with transcription of bcl-6 prevents binding of a transcription factor and histone acetylase/deacetylase complexes.
26. The method of claim 25, wherein the compound is N,N'-hexamethylene bisacetamide (HMBA) or trichostatin.
27. A method of treating lymphoma comprising decreasing BCL-6 levels in cells comprising the method of claim 24.

FIGURE 1

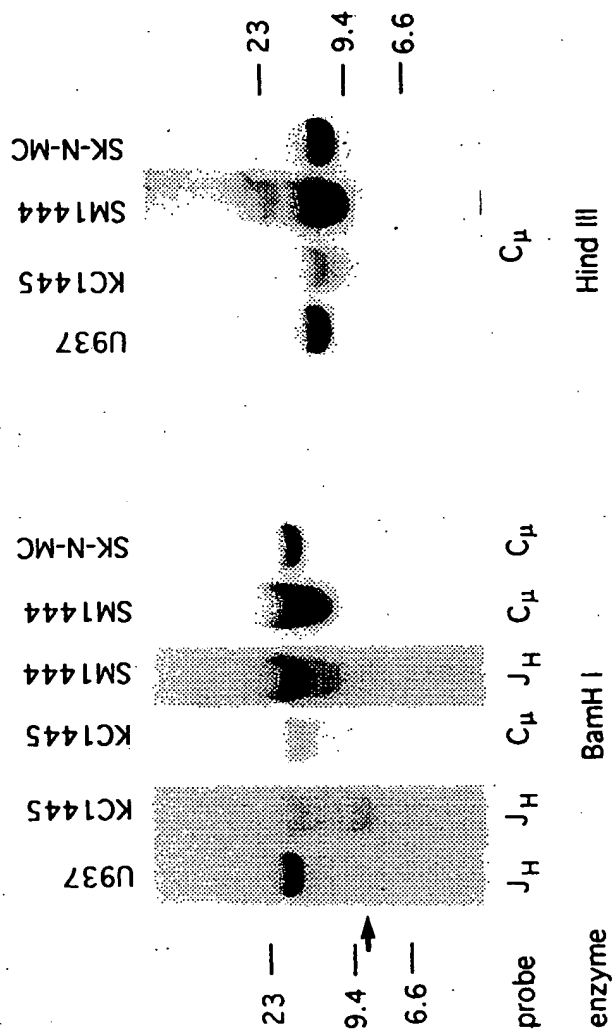
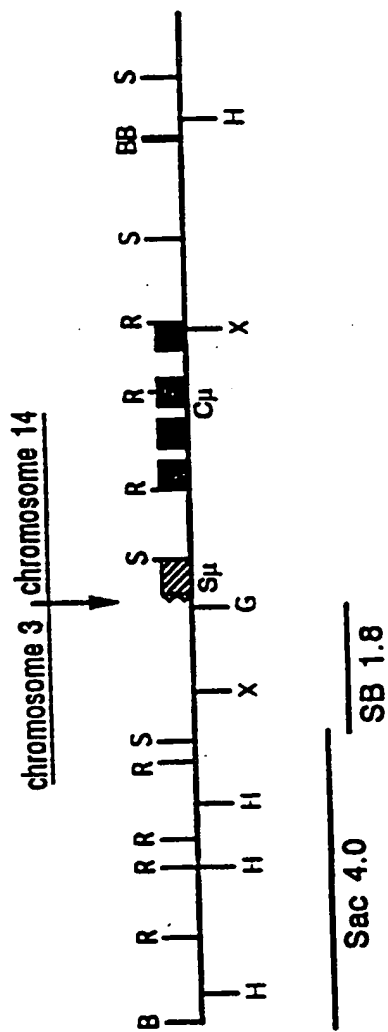


FIGURE 2

SM-71



KC-51

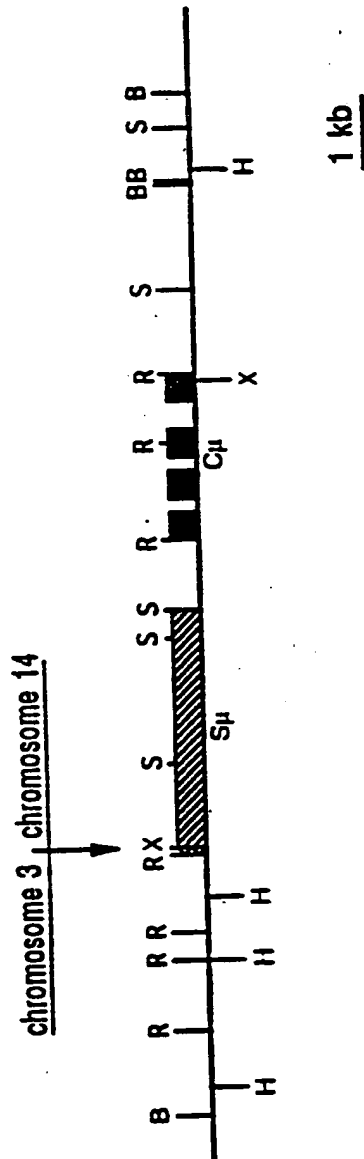


FIGURE 3A

FIGURE 3B

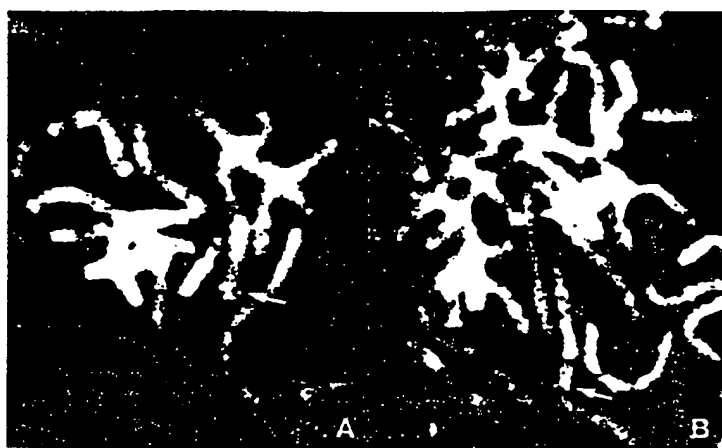


FIGURE 4A

FIGURE 4B

FIGURE 4C

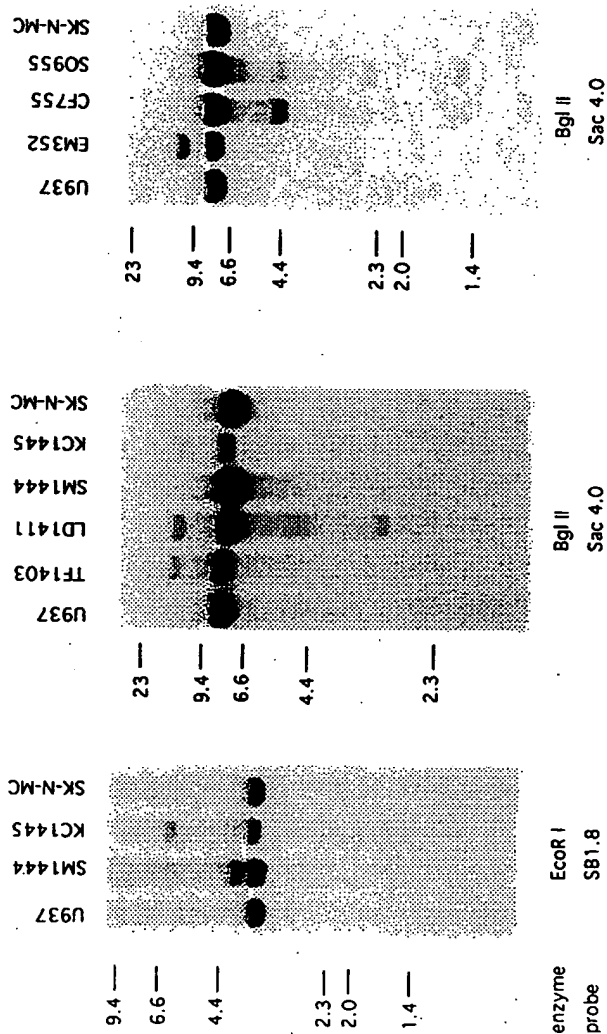


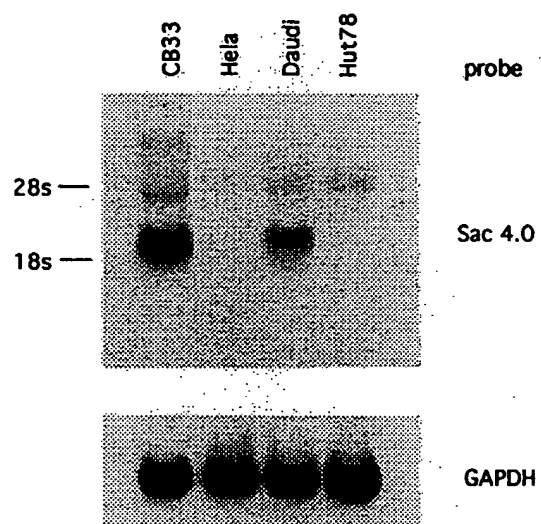
FIG. 5

FIG. 6

Map of Human *BCL-6* Locus

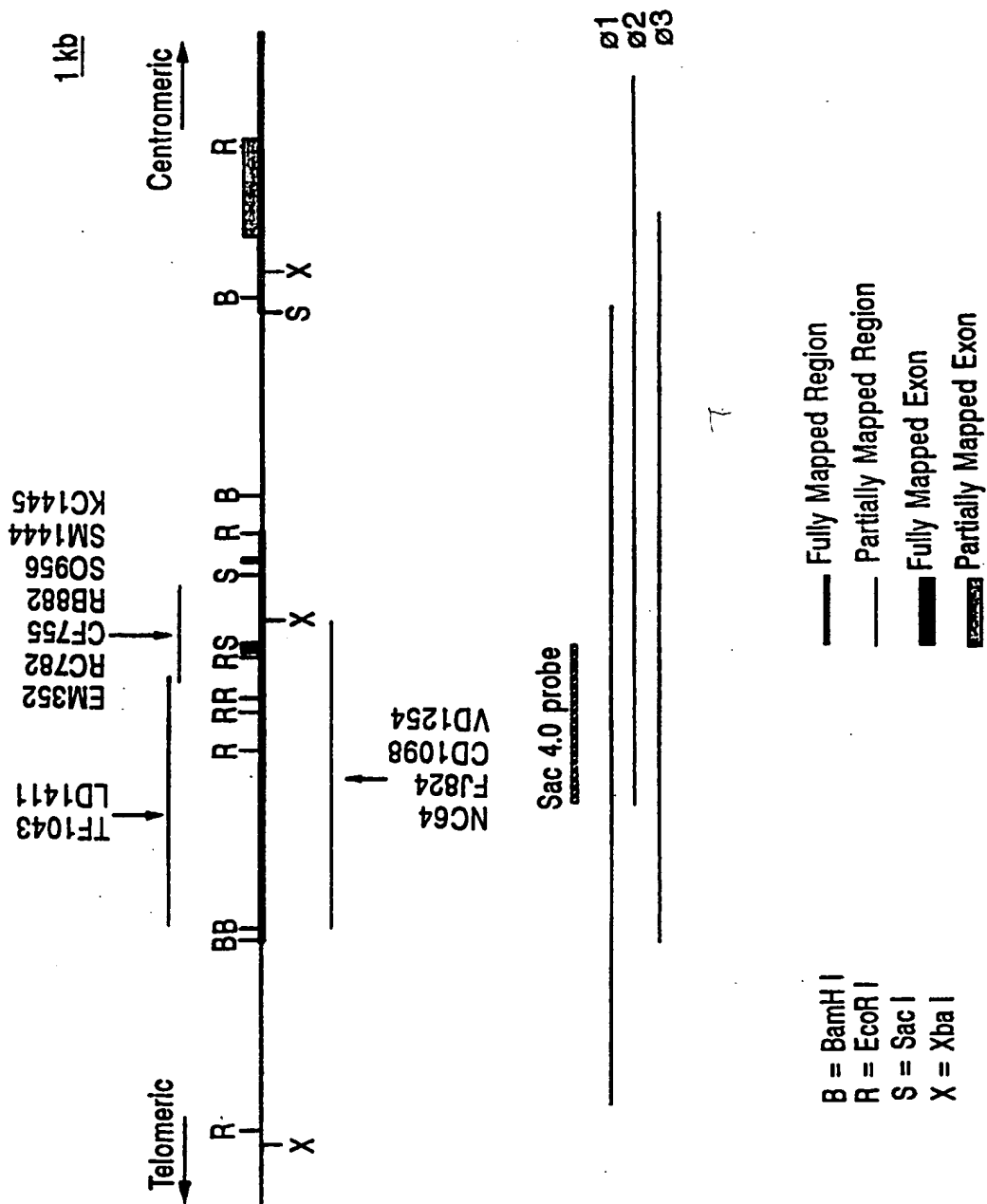
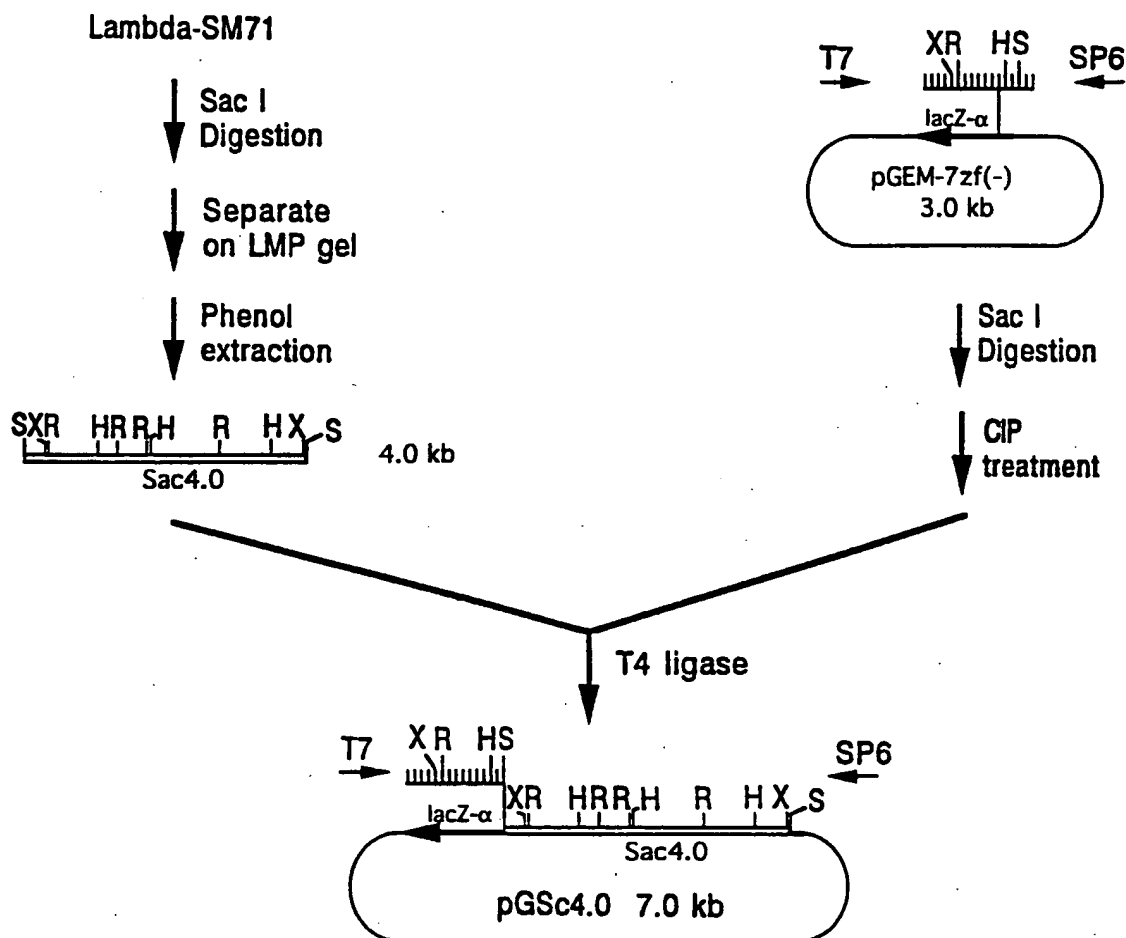


FIG. 7



Lambda-SM71 = a recombinant Lambda phage clone
containing *Bcl-6* breakpoint

H = Hind III
R = EcoR I
S = Sac I
X = Xho I

FIG. 8

Lambda-B31

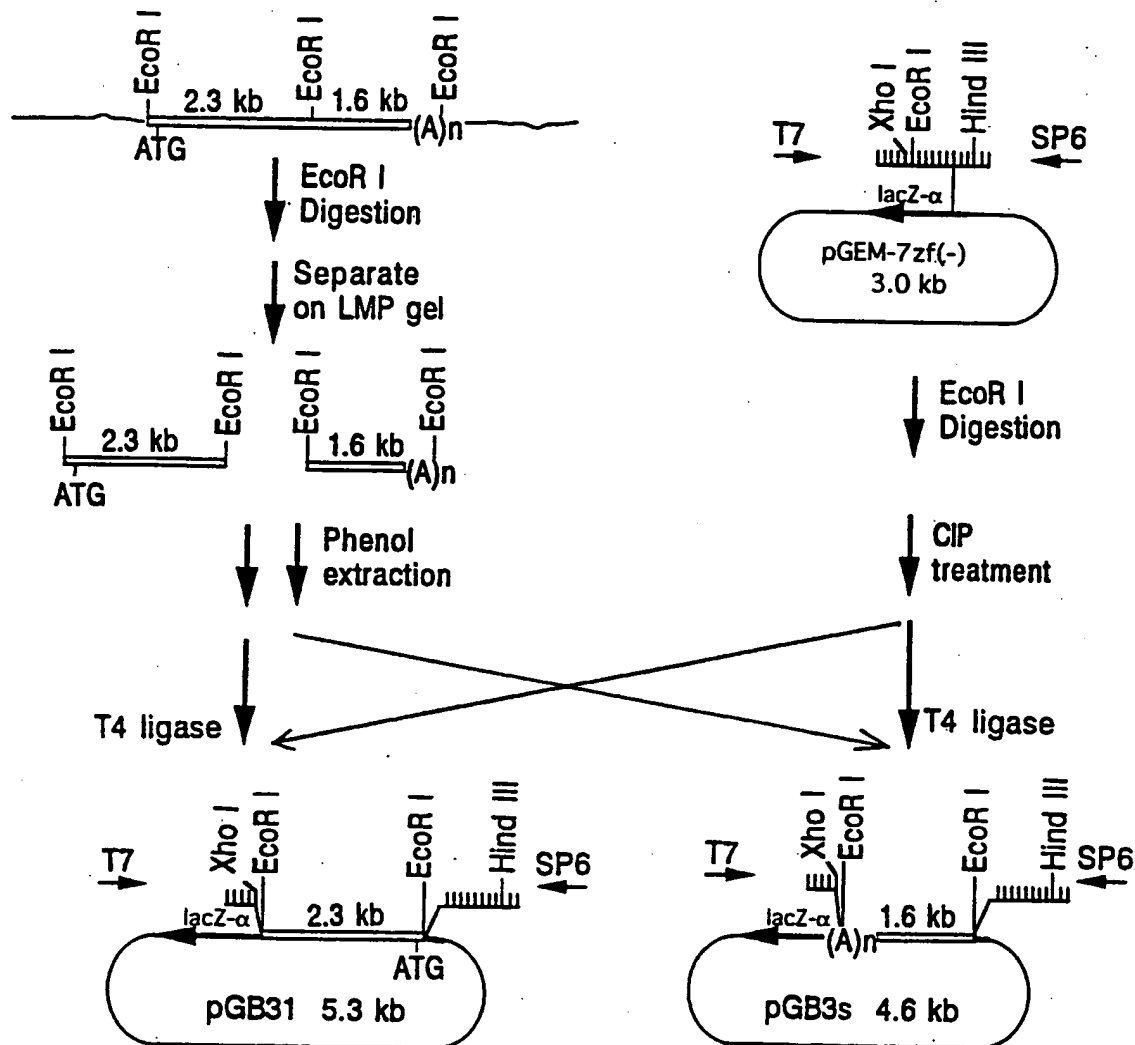


FIGURE 10A

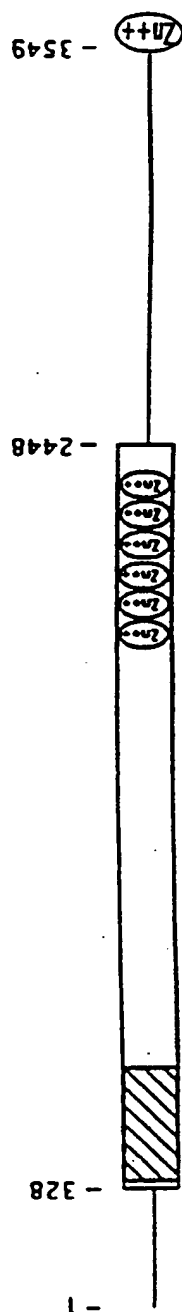


FIGURE 10B

1 MASPADSCIQ FTRHASDVLL NLNRLRSRDI LTDVVIVVSR EQFRAHKTVL
51 MACSGLFYSI FTDQLKCNLS VINLDPEINP EGFCILLDFM YTSRLNLREG
101 NIMAVMATAM YLQMEHVVDI CRKFIKASEA EMVSAIKPPR EEFLNSRMLM
151 PQDIMAYRGR EVVENNLPLR SAPGCESRAF APSLYSGLST PPASYSMYSH
201 LPVSSLLFSD EEFRDVRMPV ANPFPKERAL PCDSARPVPG EYSRPTLEVS
251 PNVCHSNIYS PKETIPEEAR SDMHYSVAEG LKPAAPSARN APYFPCDKAS
301 KEEERPSSD EIALHFEPN APLNRKGLVS PQSPQKSDCQ PNSPTEACSS
351 KNACILQASG SPPAKSPTDP KACNWKKYKF IVLNSLNQNA KPGGPEQAEI
401 GRLSPRAYTA PPACQPPMEP ENLDLQSPK LSASGEDSTI PQASRLNNIV
451 NRSMTGSPRS SSESHSPLYM HPPKCTSCGS QSPQHAEMCL HTAGPTFAEE
501 MGETQSEYSD SSCENGAFEC NECDREFSEE ASLKRHTLOT HSDKPYKCDR
551 COASFRYKGN LASHKTVHTG EKPYRENICG AQENRPANLK THTRHSGEK
601 PYKEETCGAR FVOVAHLRAH VLTHTEKPY PCETCGTRFR HLDLEKSHLR
651 THTEKPYH EKCNLHFRHK SOLRLHLROK HGAITNTKVQ YRVSATDLPP
701 ELPKAC*

FIG. 11

ZFPJS	(2-56)	DGSFVQISVRVIEQENKQREKQYCDATIDVGGGLVFKAHWSVLACCSHFFQSLYGG
KUP	(1-54)	.MDTASHBLVLEQQNMQRFEFGFCDCCTVAIGDVYFKAHRAVLAAFSNYFKMTFFI
VA55R	(1-51)	...MNSSELEIAVINGFRNSGRFCDSIVINDERINAHKLIISGASEYFSILFS
ttk	(9-63)	CWRWNHGSNLSVFDQQLHAEFTIDVTIAVEGQHUKAHKNVLSACSPYFNTLHV
ke1ch	(132-186)	QYSNEQHTARSFDAMNEMRKQKQECQVILVADVEITHAHRMVLASCSPPYFYAMFI
PLZF	(10-63)	QIQNPSHPITGEECKANQMRLAGTECDVMIMVDSQEFHAHRITVLACTSKMEILF.
BCL-6	(8-62)	CQFTRHASDVLELNENRERSRDIETIDVMIVVSREQERAHKIVLMACSGLFYSIFI
ZFPJS	(57-104)	DG..SGGSV.VBPAGF.AEIFFGLLEDEFFYTCHLALTSGNRDQVLLWARERMV...
KUP	(55-107)	HQ..TSECIKTQPTDIQPDIESYLHIMYTCKGPKQIVDHSRLEEGIRFHADYI
VA55R	(52-106)	NNFIDSNEYEVNLSHDYQSVNDLIDYIYGIPLSLTNDNVKYILSTADFLQIGSA
ttk	(64-116)	SH..PEKHPIVILKDVPSYDMKSLDEFMYRGEVSVQGERLTAFLRVAESIRIKGL
ke1ch	(187-240)	SFEESRQARITIQS.VDARALELIDYVYTATVEVNEEDNVQVMTAANLQLTDV
PLZF	(64-114)	...HRNSQHYTIDF.LSPKTEQQILEYAYTATLQAKAEDDDLEYYAABIETIYEL
BCL-6	(63-117)	DQLKCNLSVINDEEINPEGCIIDEFMYTSRLNREGNIMAVMATIAMYLQMEHV

FIGURE 12

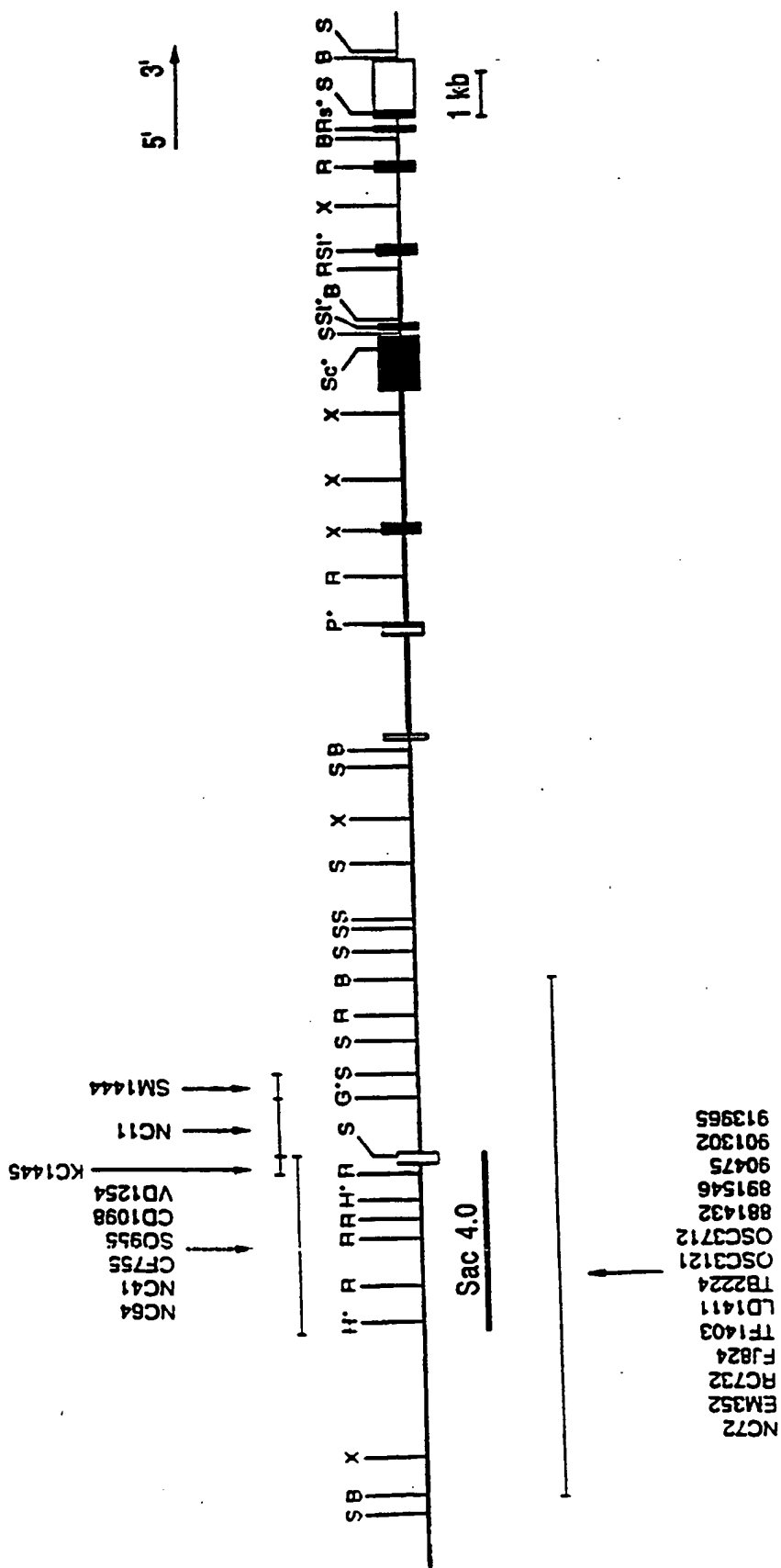


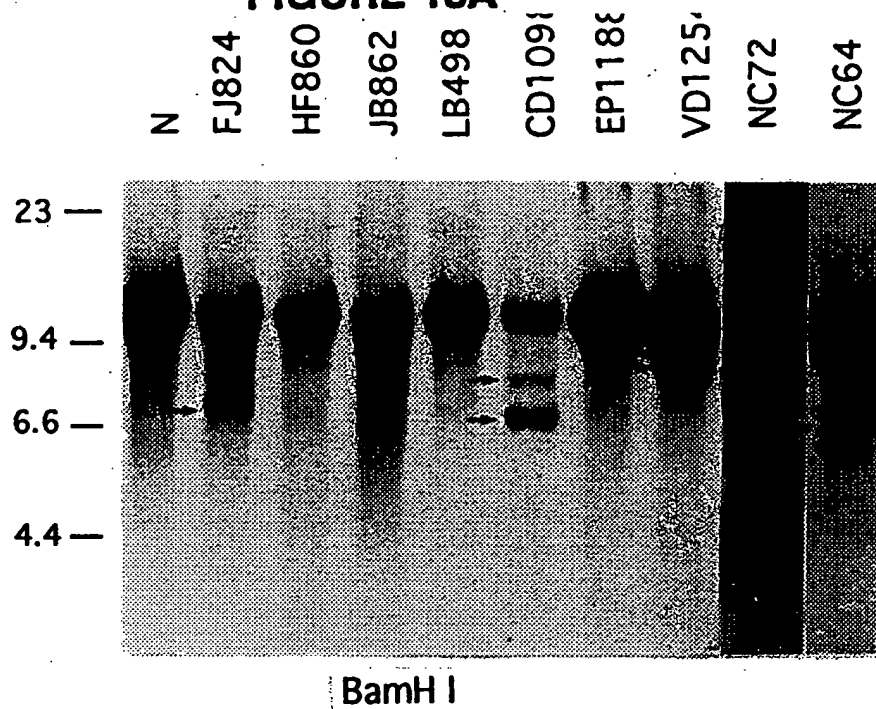
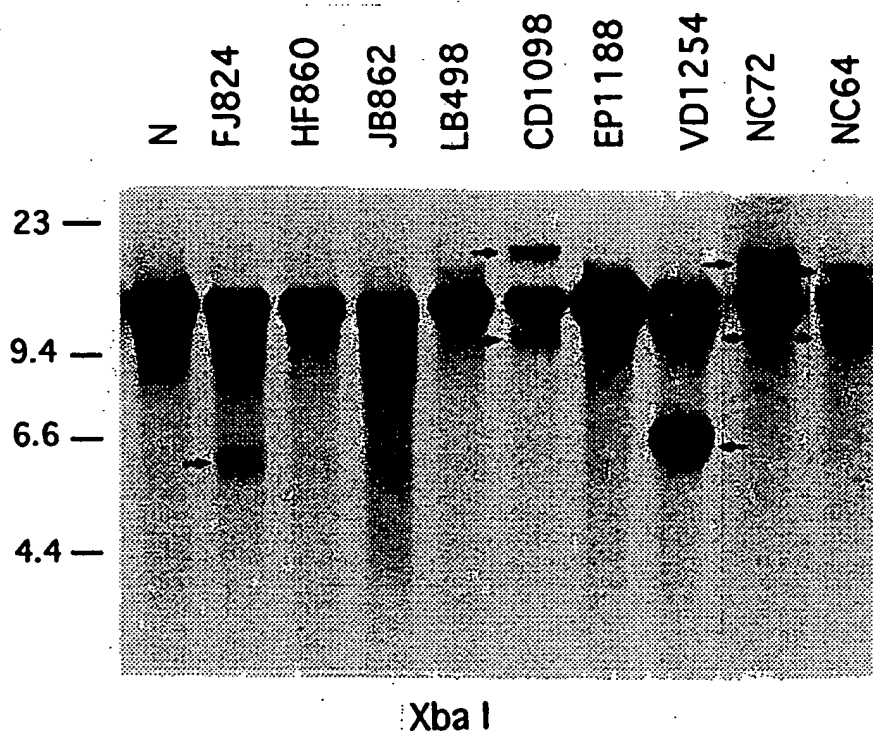
FIGURE 13A**FIGURE 13B**

FIGURE 14C

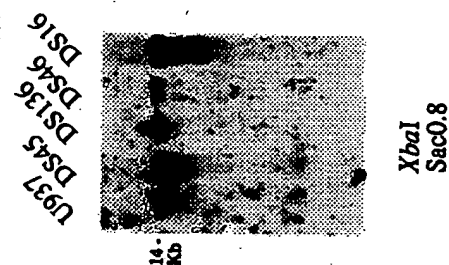


FIGURE 14B

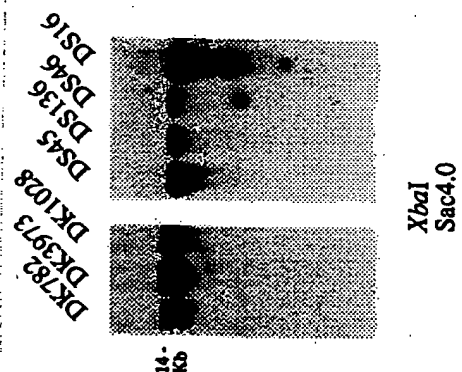


FIGURE 14A

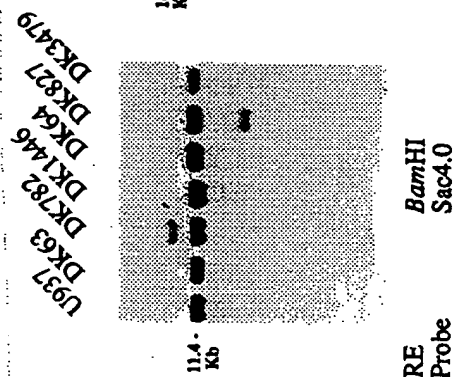


FIGURE 15

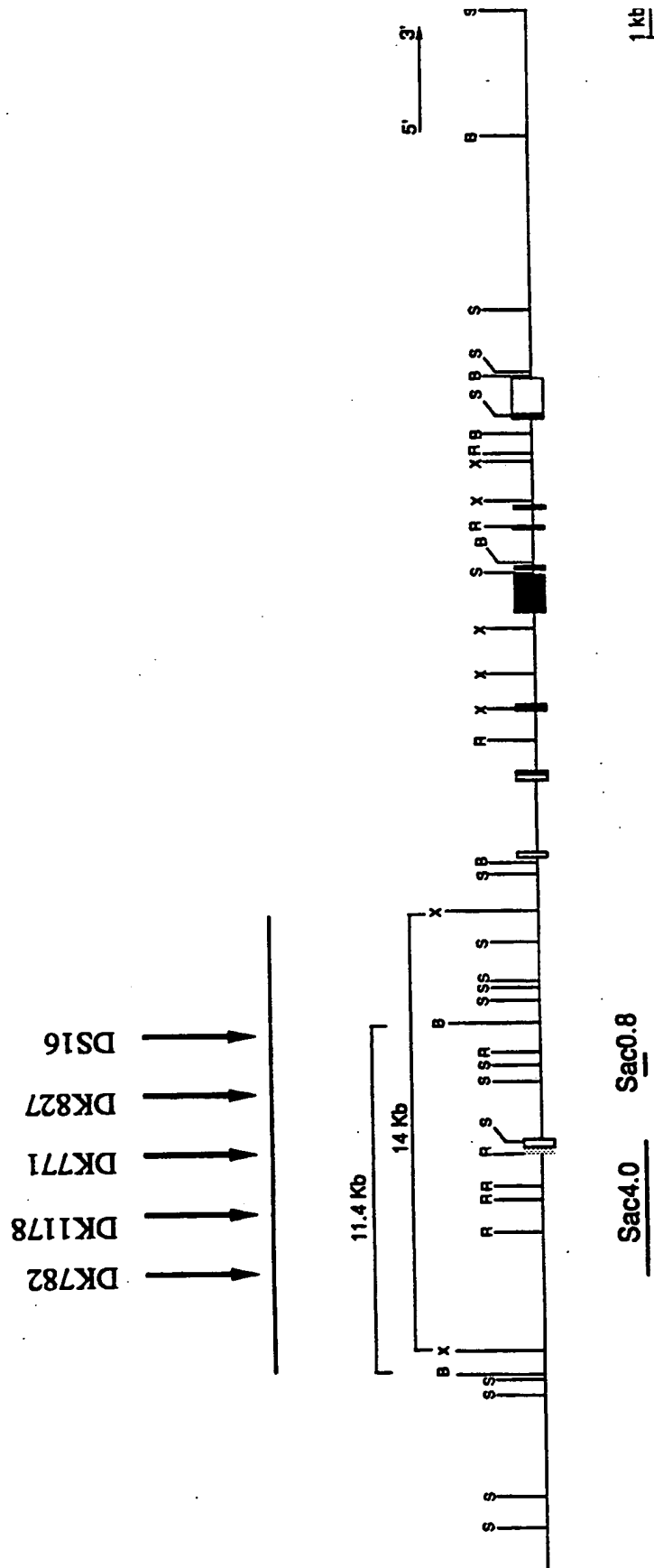


FIGURE 16C

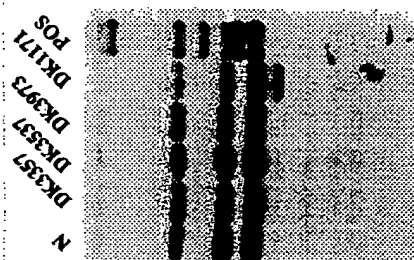


FIGURE 16B

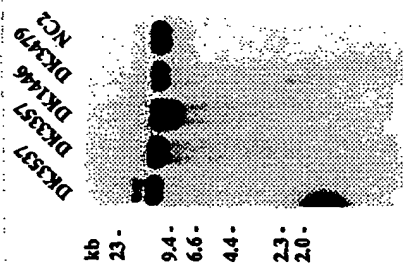


FIGURE 16A

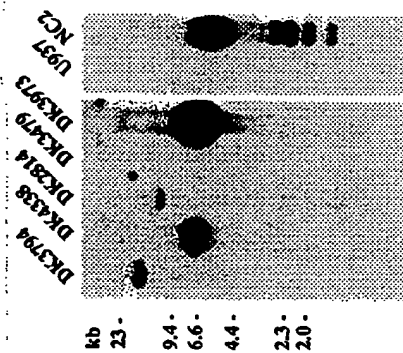
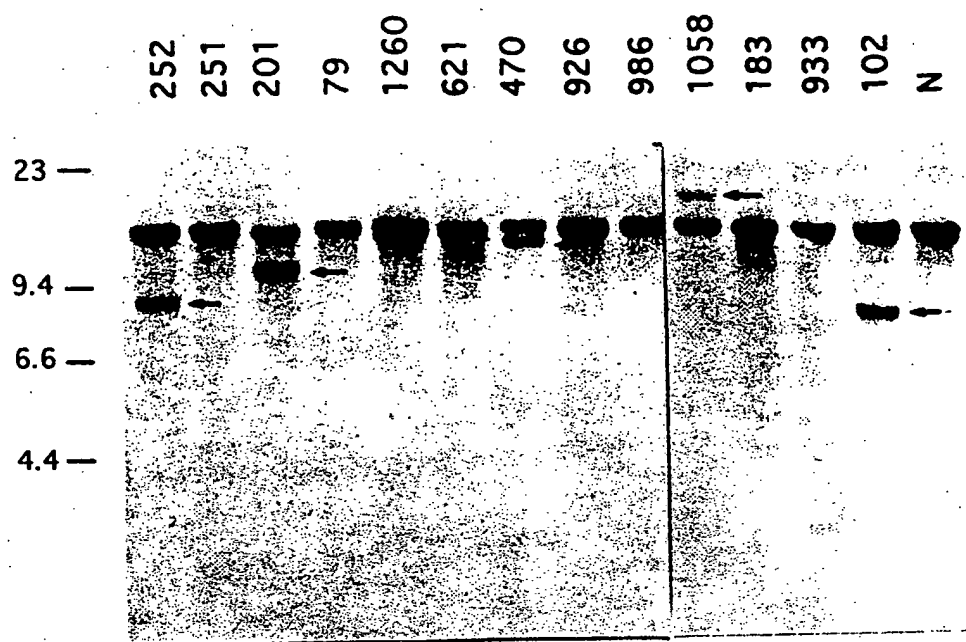
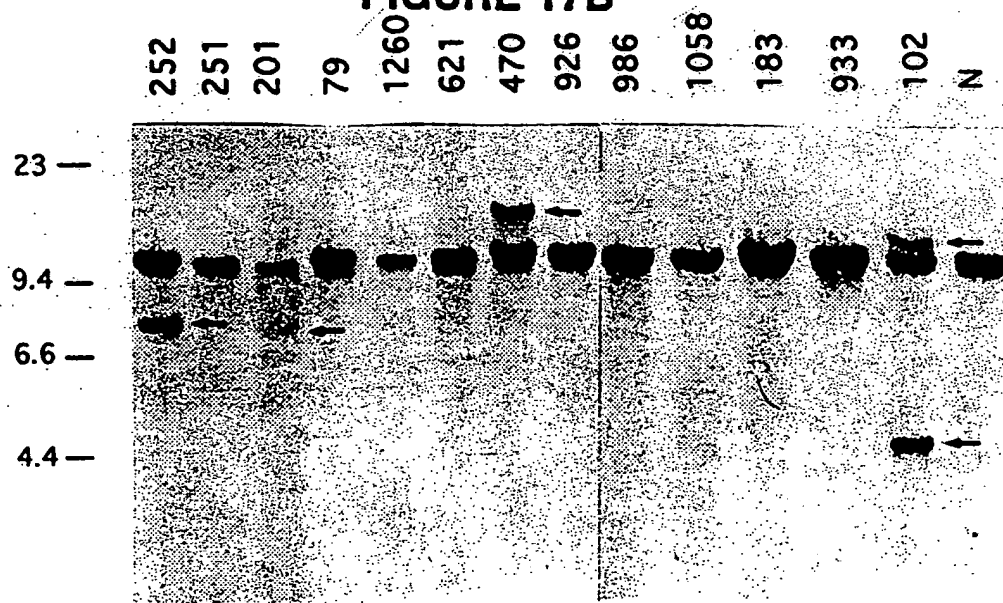


FIGURE 17A

Xba I

FIGURE 17B

BamH I

FIGURE 18A

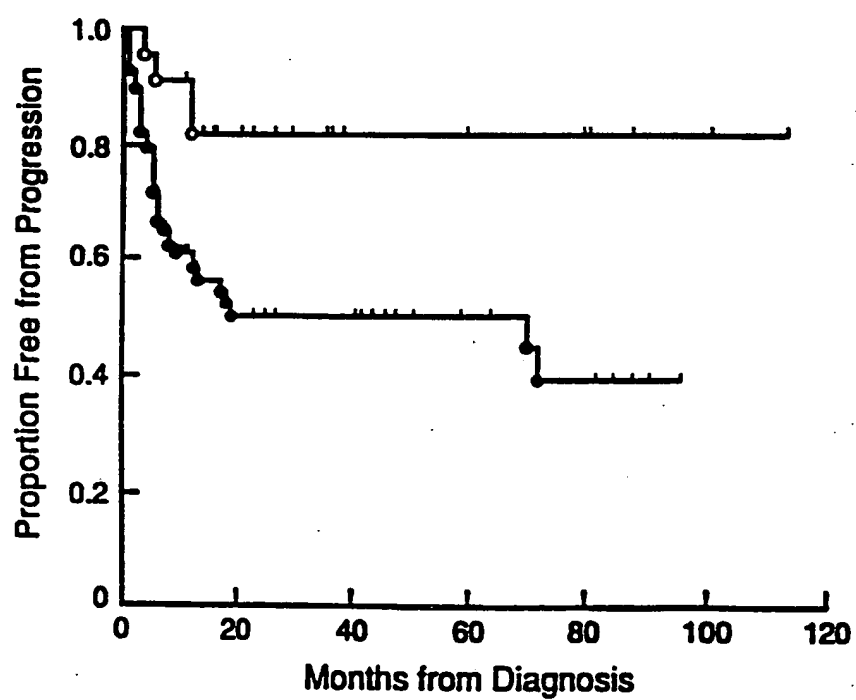


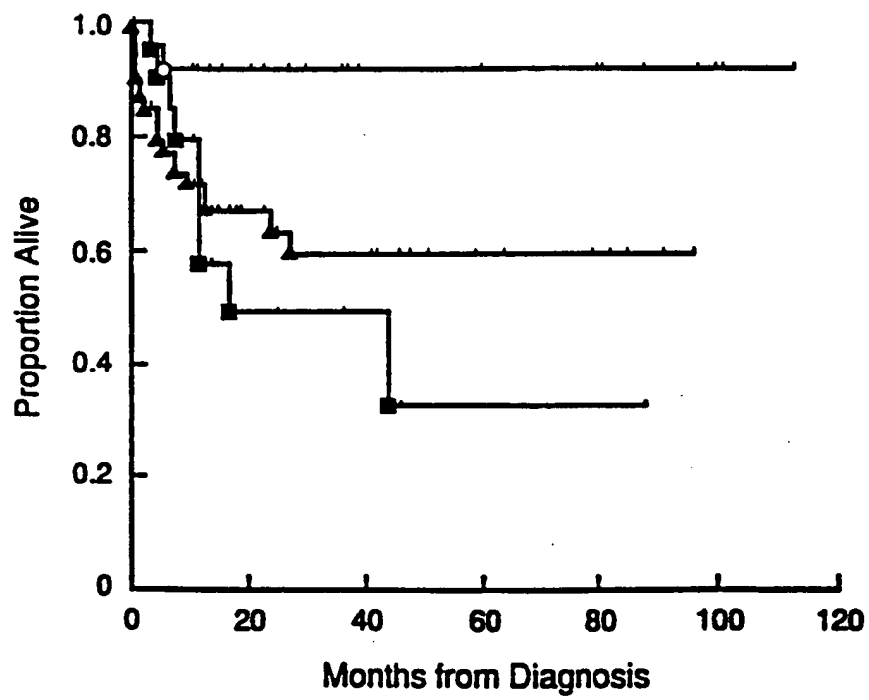
FIGURE 18B

FIGURE 19A

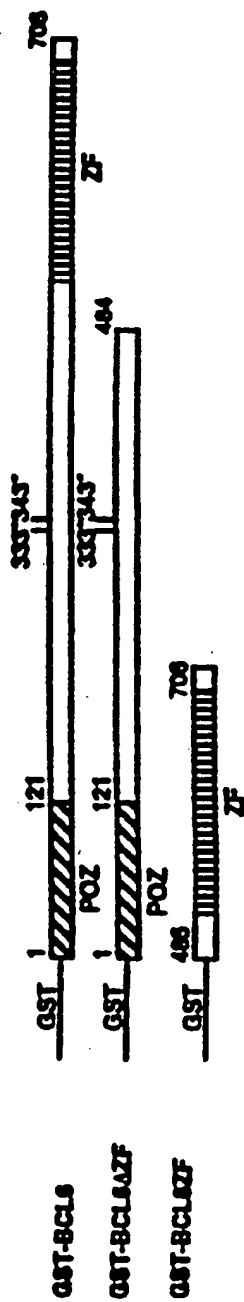


FIGURE 19B

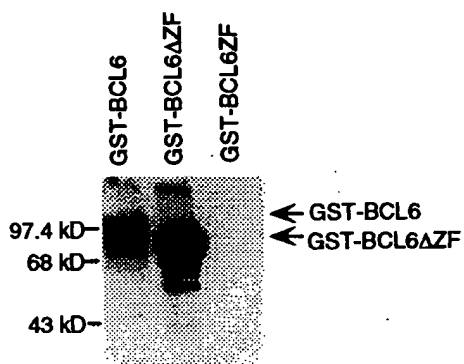


FIGURE 19C

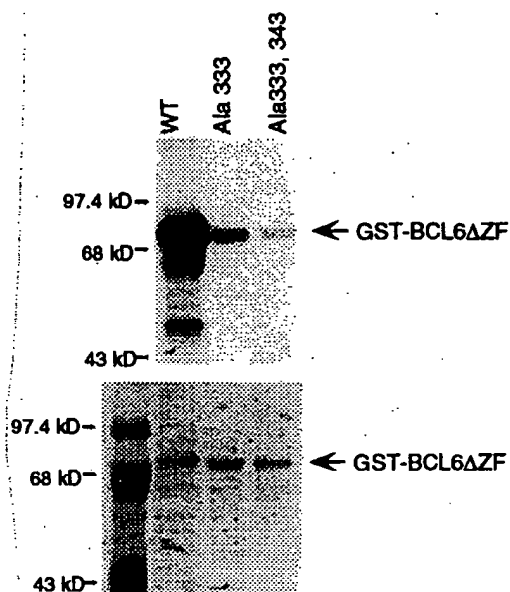


FIGURE 20A

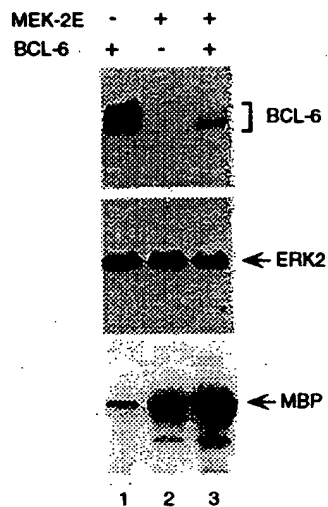


FIGURE 20B

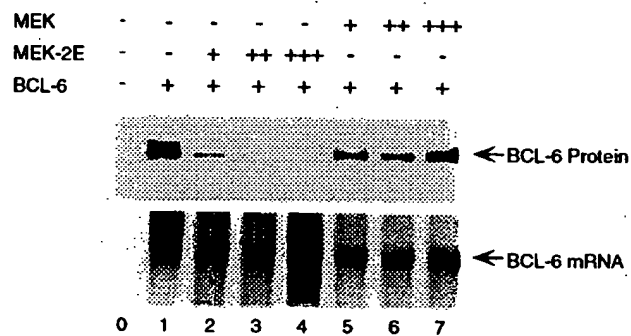


FIGURE 20C

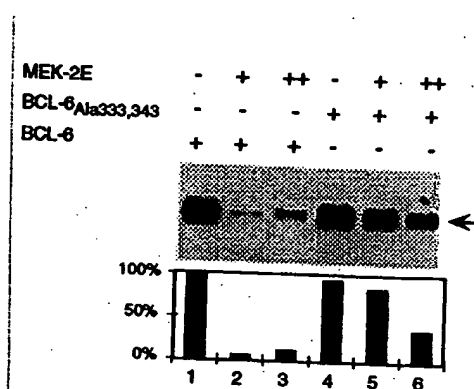


FIGURE 20D

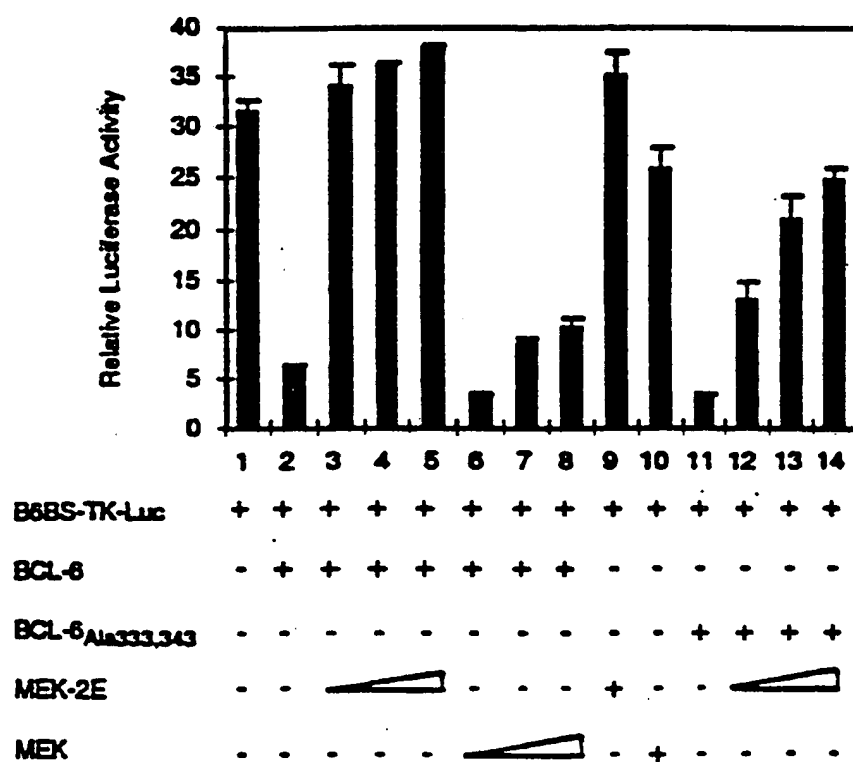


FIGURE 21A

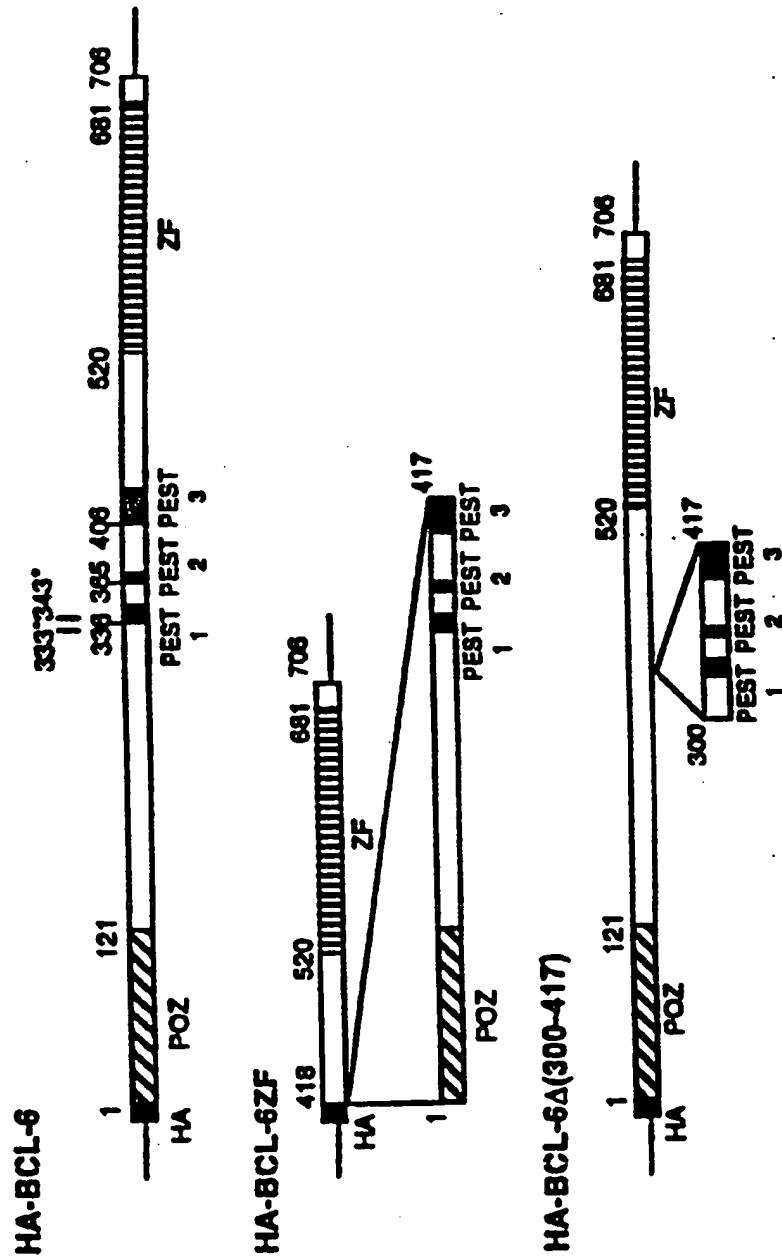


FIGURE 21B

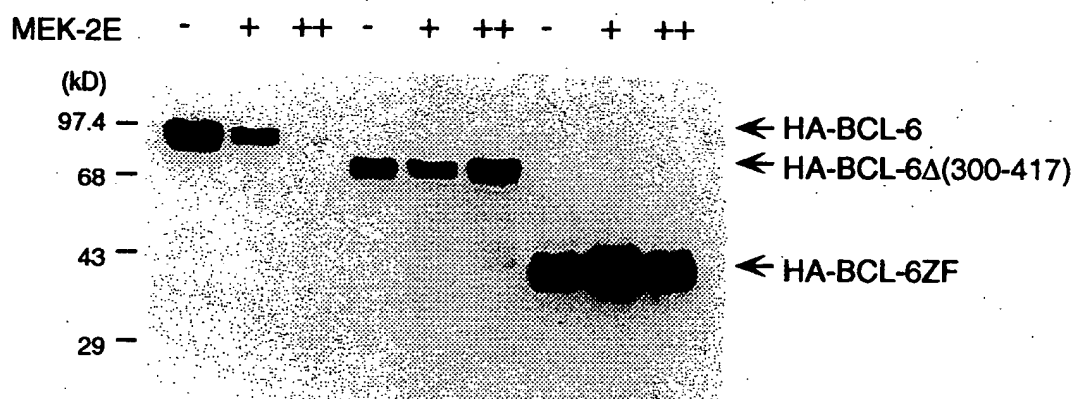


FIGURE 22A

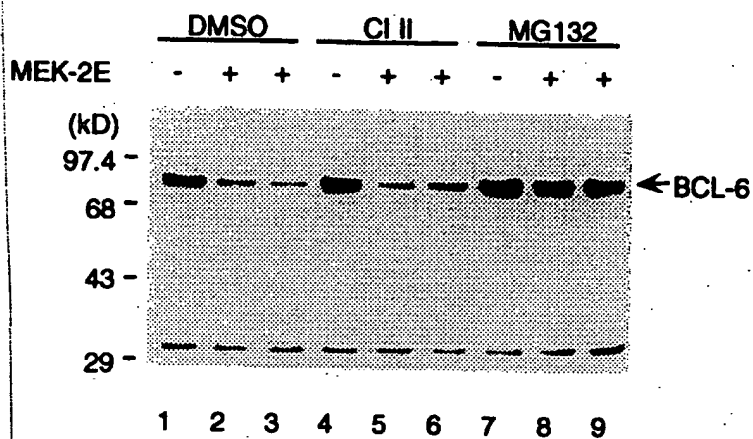


FIGURE 22B

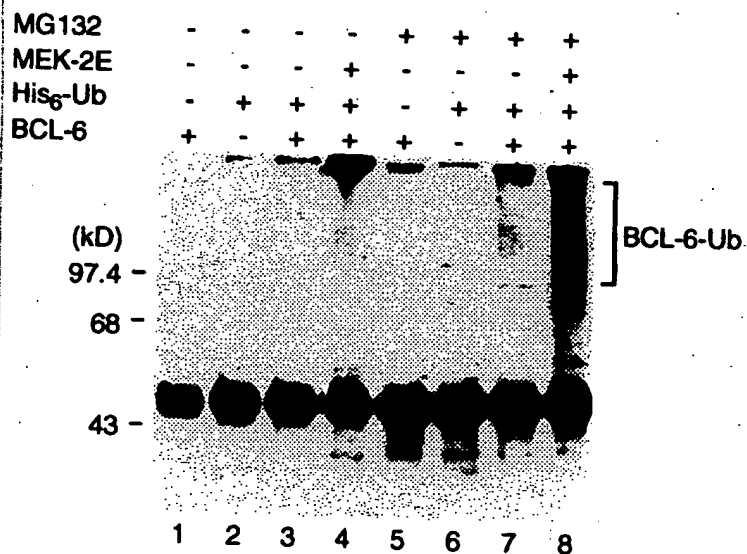


FIGURE 23A

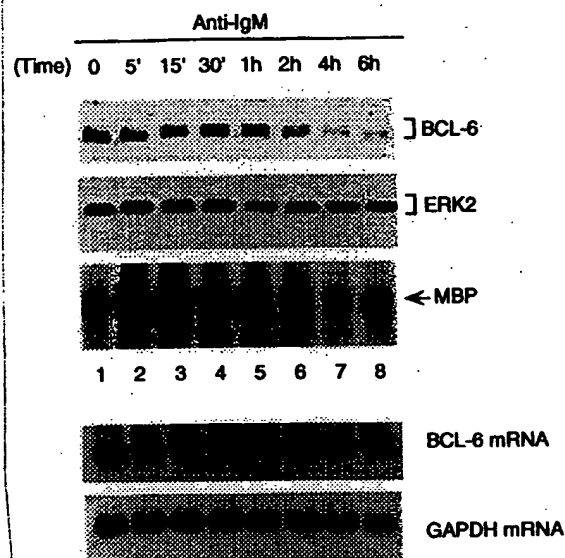


FIGURE 23B

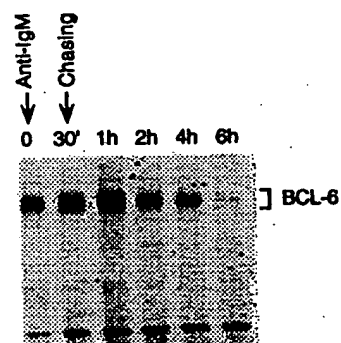


FIGURE 23C

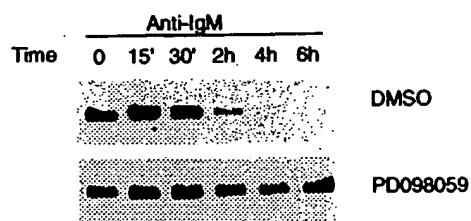


FIGURE 23D

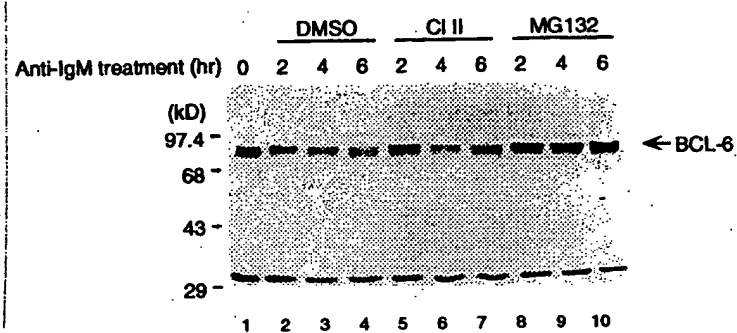
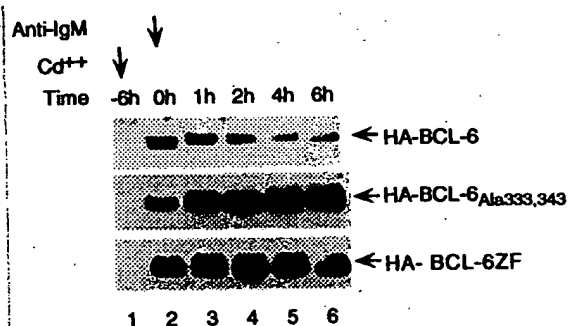



FIGURE 23E



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14703

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : A61K 31/00, 38/19, 38/20, 38/45, 39/395 US CL : 428/85.1, 94.1, 130.1; 514/1 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/85.1, 94.1, 130.1; 514/1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	LIN et al. Anti-IgM-induced growth inhibition and apoptosis are independent of ornithine decarboxylase in Ramos cells. Experimental Cell Research. 25 November 1997, Vol. 237, No. 1, pages 231-241, especially pages 231-232.	1, 5-9, 12, 13 ----- 14-18, 21-23
X — Y	MORIYAMA et al. BCL-6 is phosphorylated at multiple sites in its serine- and proline-clustered region by mitogen activated protein kinase (MAPK) <i>in vivo</i> . Oncogene. 22 May 1997, Vol. 14, No. 20, pages 2465-2474, especially page 2465.	1, 2, 4 ----- 3
X	US 4,863,727 A (ZIMMERMAN et al.) 05 September 1989, see claim 8.	14, 16, 19, 20
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or can... considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family	
Date of the actual completion of the international search 09 SEPTEMBER 1999		Date of mailing of the international search report 09 November 1999 (09.11.99)
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer DAVID S. ROMEO  Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14703

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEMMELE et al. Differentiation of Burkitt lymphoma cells by hexamethylenbisacetamide. Molecular Biology Reports. 1989, Vol. 13, pages 151-157, especially page 151.	24-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14703

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE

search terms: lymphoma, hodgkin's, anti-IgM, IL-2, IL-6, TNF, HMBA, trichostatin, bcl-6, germinal centers, large cell lymphoma, laz3, bcl5

SQ Sequence 706 AA;

Query Match	100.0%;	Score 3793;	DB 3;	Length 706;
Best Local Similarity	100.0%;	Pred. No. 9.8e-284;		
Matches 706;	Conservative 0;	Mismatches 0;	Indels 0;	Gaps 0

Qy	1	MASPADSCIOFRRHSDVILNINLRSDILTDVIVISBEOFAHNTVIMASCSG.FY51	60
Db	1	MASPADSCIOFRRHSDVILNINLRSDILTDVIVISBEOFAHNTVIMASCSG.FY51	60
Qy	61	PTDOLKCNLSVINDPEINBPGCILLDPMTTSLNLRBGINAVATANTYLOMEHVDT	120
Db	61	PTDOLKCNLSVINDPEINBPGCILLDPMTTSLNLRBGINAVATANTYLOMEHVDT	120
Qy	121	CRKFIVASBAWVAIICEPREEFILNSRLMPODIMAARGREVENNPLJLASAGCESRAF	180
Db	121	CRKFIVASBAWVAIICEPREEFILNSRLMPODIMAARGREVENNPLJLASAGCESRAF	180
Qy	181	ASLSYGLSTPPASTYSMTSHLPVSSILFSDPEFVDMVPANPPKRALPCDSAPVPG	240
Db	181	ASLSYGLSTPPASTYSMTSHLPVSSILFSDPEFVDMVPANPPKRALPCDSAPVPG	240
Qy	241	EYSRPTLEVSPPVCHSNITSYSPKERTPEARSDMHYSABGLKPAAPARANAFFPCDDKAS	300
Db	241	EYSRPTLEVSPPVCHSNITSYSPKERTPEARSDMHYSABGLKPAAPARANAFFPCDDKAS	300
Qy	301	KEEBRPSDEIATLHPEBPAPLNRKGLVSPSPKSDCQPSPTACSSKNACTIIQASG	360
Db	301	KEEBRPSDEIATLHPEBPAPLNRKGLVSPSPKSDCQPSPTACSSKNACTIIQASG	360
Qy	361	SPPASFPDPAKCNMKTYYFTYANSLNOMAKPGSEQAEIGRLSPRAYTAPPAQOPMBP	420
Db	361	SPPASFPDPAKCNMKTYYFTYANSLNOMAKPGSEQAEIGRLSPRAYTAPPAQOPMBP	420
Qy	421	ENILDOSPTKLSASGSDSTIPQASRLANIIVRSMGTSPRSSBSHSPLVMPKCTISCOS	480
Db	421	ENILDOSPTKLSASGSDSTIPQASRLANIIVRSMGTSPRSSBSHSPLVMPKCTISCOS	480
Qy	481	OSPQHAENCLHTAGPTFAEBMEETSEYSDSCENGAFPCNECPCRSSEBASLKRHTLOT	540
Db	481	OSPQHAENCLHTAGPTFAEBMEETSEYSDSCENGAFPCNECPCRSSEBASLKRHTLOT	540
Qy	541	HSDKPYKRCOASPFYKGNLASHKTVHTGEKPRCNCGAQPNRPNLTKTHRIHSGEK	600
Db	541	HSDKPYKRCOASPFYKGNLASHKTVHTGEKPRCNCGAQPNRPNLTKTHRIHSGEK	600
Qy	601	PYKCETCGARFVOVAHLRAHVILHTGEKPYCEICGNFRHLQTLKSHLHITGEKPYHC	660
Db	601	PYKCETCGARFVOVAHLRAHVILHTGEKPYCEICGNFRHLQTLKSHLHITGEKPYHC	660
Qy	661	EKNHAFRRKSOURLHROGAILNTYVOYKVSATDLPPLPAC	706
Db	661	EKNHAFRRKSOURLHROGAILNTYVOYKVSATDLPPLPAC	706

Accession	Protein	Gene	Organism	Source	Reference
AA029640	Human bcl-6 transcriptional repressor.	BCL6	Homo sapiens	Human	1
AA029640	Human bcl-6 transcriptional repressor; germinal centre formation; Tn-2 mediated antibody affinity maturation; apoptosis regulator; chromosome 3q27; lymphoma; acute lymphoblastic leukaemia; post-transplant lymphoproliferative disorder; expression inhibition antisense therapy.	BCL6	Homo sapiens	Human	1

XX	Homo sapiens.
OS	
XX	US6140125-A.
FN	
XX	
XX	31-OCT-2000.
PD	
XX	
XX	15-OCT-1999; 99US-00418640.
PF	
XX	15-OCT-1999; 99US-00418640.
XX	
XX	(ISIS-) ISIS PHARM INC.
PA	
XX	Taylor JK, Cowseart LM;
FI	
XX	WPI; 2001-048959/06.
DR	
DR	N-PSDB; AAC81137.
XX	
XX	Antisense compounds which specifically hybridize with and inhibit human
PT	bcl-6 expression, useful for treating bcl-6 related disorders, and
PT	preventing or delaying inflammation or tumor formation.
XX	
XX	
PS	Disclosure; COL 47-52; 42pp; English.

This sequence represents human bcl-6. Bcl-6 (also known as B-cell CLL/lymphoma 6, zinc finger protein 51 and Laz3) is a sequence-specific DNA-binding transcriptional repressor. The bcl-6 gene is expressed in germinal centre B- and T- cells and is required for germinal centre formation and Th-2 mediated antibody affinity maturation. Bcl-6 may also play a role in the regulation of apoptosis. The bcl-6 gene is located on chromosome 3q27, a region which undergoes a high frequency of translocation events. Such chromosomal translocations can result in aberrant forms of bcl-6, which are strongly implicated in the pathogenesis of several types of lymphoma, and have also been reported in acute lymphoblastic leukaemia and post-transplant lymphoproliferative disorders. The invention relates to antisense oligonucleotides targeted to the human bcl-6 gene, which inhibit its expression. A series of oligonucleotides (AA081144-C81223) were designed to target different regions of the human bcl-6 mRNA, and were analysed for their effect on bcl-6 mRNA levels by quantitative real-time PCR. The oligonucleotides of the invention are useful for diagnosis, prevention and treatment of conditions associated with aberrant forms of bcl-6, such as lymphomas, acute lymphoblastic leukaemia and post-transplant lymphoproliferative disorders

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Query Match Similarity      100.0%; Score 3793; DB 4; Length 706;
Best Local Similarity      100.0%; Pred. No. 9.8e-284;
Matches      706; Conservative      0; Mismatches      0; Indels      0; Gaps      0

QY      1 MASPADSCIQFRRHASDVILNINLRSLRLITDVIVVRSREQFRAKTVLMACSGLFYSI      60
DB      1 MASPDSCIQFRRASDVILNINLRSLRLITDVIVVRSREQFRAKTVLMACSGLFYSI      60
QY      61 FTDOJKCNLSVINLDPPEINBEGFCILDDMYTSRLNLRGNTINMAVATANTYLOMEHVDT      120
DB      61 FTDOJKCNLSVINLDPPEINBEGFCILDDMYTSRLNLRGNTINMAVATANTYLOMEHVDT      120
QY      121 CRKFTKASEAVNVSAIKPPREEFILNSRLMLPODIMA YRGVEVENNLPLRSAPGCSBRAF      180
DB      121 CRKFTKASEAVNVSAIKPPREEFILNSRLMLPODIMA YRGVEVENNLPLRSAPGCSBRAF      180
QY      181 APLSYSGLSTPPASYSMTSHLTVSSLLFEDDEFRDVDMVYANFPFKERALLPCDSARVPVG      240
DB      181 APLSYSGLSTPPASYSMTSHLTVSSLLFEDDEFRDVDMVYANFPFKERALLPCDSARVPVG      240
QY      241 EKSRRPLTVSPVVCNSNITSPKRTTPEARSMDHTSVAAGLKPAAASARAAAPFPCKKAS      300
DB      241 EKSRRPLTVSPVVCNSNITSPKRTTPEARSMDHTSVAAGLKPAAASARAAAPFPCKKAS      300
QY      301 KKEERPSSEDEALHPPEPNALPINKGLVSPSPQKSDCQPNSPTEACSSKXNACILQASG      360
DB      301 KKEERPSSEDEALHPPEPNALPINKGLVSPSPQKSDCQPNSPTEACSSKXNACILQASG      360

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DB 301 KEERERSSDEI...
 QY 361 SPKASPTDPA...
 DB 361 SPKASPTDPA...
 QY 421 ENIDLOSP...
 DB 421 ENIDLOSP...
 QY 481 OSPOHAE...
 DB 481 OSPOHAE...
 QY 541 HSDKPYK...
 DB 541 HSDKPYK...
 QY 601 PYKCTGCA...
 DB 601 PYKCTGCA...
 QY 661 EKCNLH...
 DB 661 EKCNLH...
 RESULT 3
 ADL82847 standard; protein; 706 AA.
 ADL82847:
 17-JUN-2004 (first entry)
 Human PRO26296, SEQ ID 49.
 Immunosuppressive; Cytostatic; Antiarthritic; Antirheumatic; Antianemic;
 Antiallergic; Muscular; Neuroprotective; Nephrotoxic; Antiinflammatory;
 Gene Therapy; PGO; B cell related disorder; cancer;
 Immune-mediated inflammatory disease; human.
 Homo sapiens.
 WO2004024097-A2.
 25-MAR-2004.
 15-SEP-2003; 2003WO-US029097.
 16-SEP-2002; 2002US-0411392P.
 (GETH) GENENTECH INC.
 Chiu H, Clark H, Dennis K, Fong S, Schoenfeld JR, Wood WI;
 Wu TD;
 WPI; 2004-323389/30.
 N-PSDB; ADL82846.
 New PRO polypeptide, useful for diagnosing and treating a B cell related
 disorder, e.g. Burkitt's lymphoma, rheumatoid arthritis, autoimmune
 mediated hemolytic anemia, myasthenia gravis or ankylosing spondylitis.
 Claim 10; Fig 49; 695bp; English.
 The present invention relates to PRO proteins and their coding sequences.
 The PRO proteins are useful for diagnosing and treating a B cell related
 disorder, e.g. X-linked infantile hypogammaglobulinemia, polysaccharide
 antigen unresponsiveness, selective IgA deficiency, selective IgM
 deficiency, selective deficiency of IgG subclasses, immunodeficiency with
 hyper IgM, transient hypogammaglobulinemia of infancy, Burkitt's
 lymphoma, intermediate lymphoma, follicular lymphoma, type II

CC hypersensitivity, rheumatoid arthritis, autoimmune mediated haemolytic
 CC anaemia, myasthenia gravis, hypoadrenocorticism, glomerulonephritis, or
 CC ankylosing spondylitis. The PRO proteins are also useful for preparing a
 CC medicament for treating a condition that is responsive to the PRO
 CC protein, e.g. cancer or immune-mediated inflammatory diseases. The PRO
 CC coding sequences are useful as hybridization probes in chromosome and
 CC gene mapping, in preparing PRO proteins, or in generating transgenic
 CC animals or knockout animals, which if turn are useful in the development
 CC and screening of therapeutically useful reagents.
 XX
 SO Sequence 706 AA;
 Query Match 100.0%; Score 3793; DB 8; Length 706;
 Best Local Similarity 100.0%; Pred. No. 9.8e-284;
 Matches 706; Conservative 0; Mismatches 0; Indels 0; Gaps 0;
 QY 1 MASPADSC...
 DB 1 MASPADSC...
 QY 61 FTDLKCN...
 DB 61 FTDLKCN...
 QY 61 FTDLKCN...
 DB 61 FTDLKCN...
 QY 121 CRKFIK...
 DB 121 CRKFIK...
 QY 121 CRKFIK...
 DB 121 CRKFIK...
 QY 181 APSLYSG...
 DB 181 APSLYSG...
 QY 241 EYSRPT...
 DB 241 EYSRPT...
 QY 301 KEERER...
 DB 301 KEERER...
 QY 361 SPKAS...
 DB 361 SPKAS...
 QY 421 ENIDLO...
 DB 421 ENIDLO...
 QY 481 OSPOH...
 DB 481 OSPOH...
 QY 541 HSDKPY...
 DB 541 HSDKPY...
 QY 601 PYKCTG...
 DB 601 PYKCTG...
 QY 661 EKCNLH...
 DB 661 EKCNLH...
 RESULT 4
 ADL14017 standard; protein; 706 AA.
 ADL14017:
 21-OCT-2004 (first entry)

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